Role of Natural Killer Cells in Epstein-Barr Virus Infection

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The only aids used for composing this dissertation are those stated therein.

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1. SUMMARY

Epstein-Barr virus (EBV) latently infects more than 90% of adults worldwide and is associated with epithelial and B cell tumors. Primary EBV infections in toddlers are mostly asymptomatic, but may develop into the symptomatic infectious mononucleosis (IM) in adolescents and adults. T cell responses are known to be essential for the lifelong immune control of this tumor virus, however the role of natural killer (NK) cells in EBV infection has not been extensively investigated yet. NK cells are composed of several subsets and specific subsets have been shown to preferentially expand during acute viral infections other than EBV in humans. Based on reports of age-related changes in NK cell subsets distribution and function, we postulated that healthy individuals older than 5 years of age exhibit an impaired NK cell immune response against EBV, leading to T cell-mediated immunopathology and IM.

In the work presented here, we longitudinally assessed NK cell responses in pediatric IM patients. Our results indicate that children and teenagers with IM exhibit the classical T cell features observed in adults. Acute IM drives the preferential proliferation and accumulation of early-differentiated CD56dim NKG2A+ KIR- NK cells. Moreover, only this NK cell subset further maturates during the first month of IM and remains at increased frequency for up to 6 months compared to controls. Additionally, we demonstrate that this NK cell subset specifically recognizes and responds to EBV-infected B cells with viral reactivation. We also found that the frequencies and absolute numbers of this NK cell subpopulation in peripheral blood decrease during the first decade of life. Finally, we did not observe any age-related changes in the NK cell-mediated response against EBV-transformed B cells *in vitro*.

This is the first report of an expanding human NK cell subset that directly recognizes virus-infected cells *in vitro*. Our novel findings support the hypothesis that the early NK cell-mediated immune control of EBV lytic replication is quantitatively impaired after the first decade of life and might therefore predisposes to IM. This work provides insight into a protective NK cell response in EBV infection, which might be exploited for the development of novel immunotherapies against this tumor virus.

2. ZUSAMMENFASSUNG

Das Epstein-Barr Virus (EBV) etabliert eine lebenslang persistierende Infektion in mehr als 90% der erwachsenen Bevölkerung weltweit und ist mit bösartigen epithelialen und B-Zell-Tumoren assoziiert. Erstinfektionen mit EBV verlaufen bei Kleinkindern häufig asymptomatisch, können aber in Jugendlichen und Erwachsenen zur Entwicklung der Infektiöse Mononukleose (IM) führen. T Zell-Antworten sind wesentlich für die lebenslange Immunkontrolle dieses Tumorvirus, jedoch wurde die Rolle von natürlichen Killer (NK) Zellen in der EBV Infektion bis heute wenig untersucht. NK Zellen bestehen aus verschiedenen Subpopulationen und es konnte gezeigt werden, dass bestimmte Subpopulationen bei verschiedenen akuten viralen Infektionen im Menschen präferenziell expandieren. Aufgrund von Berichten über altersabhängige Veränderungen der Subpopulationenverteilung und der Funktion der NK Zellen schlagen wir die Hypothese vor, dass gesunde Leute, die älter als 5 Jahre sind, zeigen eine durch NK Zellen ungenügende Immunkontrolle von EBV, die zur durch T Zellen ausgelösten Immunpathologie und IM führt.

In der hier vorliegenden Arbeit wurde anhand einer longitudinalen Studie das Verhalten von NK Zellen und deren Subpopulationen in pädiatrischen Patienten mit IM untersucht. Unsere Daten zeigen, dass bei Kindern und Teenagers mit IM die klassischen T Zellen Eigenschaften von Erwachsenen beobachtet werden. Die akute IM führt zu einer spezifischen Proliferation und Akkumulation von früh differenzierten $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} NK Zellen. Außerdem wird ausschließlich diese NK Zellen Subpopulation über den ersten Monat von IM reifer und ihre Häufigkeit bleibt erhöht bis 6 Monate nach IM. Darüber hinaus können wir zeigen, dass diese Subpopulation von NK Zellen vor allem EBV infizierte B Zellen mit viraler Reaktivierung erkennt. Weiterhin belegen unsere Daten, dass Häufigkeiten und Zellzahlen dieser NK Zell-Population im peripheren Blut über das erste Lebensjahrzehnt abnehmen. Schließlich haben wir keine altersabhängige Unterschiede in NK Zellen Antworten gegen EBV transformierte B Zellen *in vitro* beobachtet.

In dieser Arbeit beschreiben wir erstmalig eine expandierende humane NK Zell Subpopulation, die *in vitro* direkt Virus infizierte Zellen erkennen kann. Unsere neue Erkenntnisse passen zur Hypothese, dass die durch NK Zellen vermittelte frühe Immunkontrolle von EBV lytischer Vermehrung nach Ablauf des ersten Lebensjahrzehntes quantitativ weniger effizient wird und somit zur Entwicklung der IM prädisponiert. Unsere Einblicke in eine schützende NK Zell-Antwort gegen die EBV Infektion könnten zur Entwicklung neuer Immuntherapien gegen dieses Tumorvirus führen.

3. INTRODUCTION

3.1. Epstein-Barr virus

3.1.1 History of EBV

The Epstein-Barr virus (EBV) belongs to the family of γ-herpesviruses and was first described in 1964 by Anthony Epstein in tissue cultures of Burkitt lymphoma biopsies (1). Later, thanks to the development of immunofluorescent tests to detect viral antigens by Gertrude and Werner Henle, it became clear that EBV infections are ubiquitous and that the virus is found widespread in humans (2). The Henles then identified EBV as the causative agent for infectious mononucleosis (IM) in 1968, when one technician from their lab suffered from this disease and further develop antibodies to this herpesvirus (3). This observation was confirmed in a prospective seroepidemiological study of university students, showing that only EBV-seronegative individuals developed IM and exhibited seroconversion (4). EBV was later detected in tissues from patients with nasopharyngeal carcinoma in the 1970s (5), non-Hodgkin lymphoma (NHL) in patients with acquired immunodeficiency syndrome (AIDS) in the early 1980s (6), and T cell and classical Hodgkin lymphomas (HL) in the late 1980s (7, 8). EBV is currently associated with several cancers both in immunocompetent and immunocompromised individuals (Table 1, adapted from (9)).

Immunocompetent host	*Immunocompromised host*
B cell malignancies	*B cell malignancies*
Burkitt lymphoma	Acquired immunodeficiency
Classical Hodgkin lymphoma	AIDS-associated B cell lymphomas
T cell malignancies	Post-transplantation lymphoproliferative disorder
Extranodal NK/T cell lymphoma, nasal type	Lymphomatoid granulomatosis
Virus-associated hemophagocytic syndrome T cell lymphomas	Methotrexate-associated B cell lymphoma
Epithelial cell malignancies	Congenital immunodeficiency
Nonglandular nasopharyngeal carcinoma	Severe combined immunodeficiency–associated B cell lymphomas
Lymphoepithelioma-like carcinoma (salivary, thymus, lungs, stomach)	Wiskott-Aldrich syndrome–associated B cell lymphomas
Breast carcinoma	X-linked lymphoproliferative disorder–associated B cell lymphomas
Hepatocellular carcinoma	*Mesenchymal malignancies*
Mesenchymal malignancies	Leiomyosarcoma
Follicular dendritic cell sarcoma	

Table 1: EBV-associated malignancies (adapted from (9))

3.1.2. EBV epidemiology

Seroepidemiologic surveys suggest that EBV infects more than 95% of the adult population worldwide (10). The prevalence of EBV antibodies varies in different regions of the world depending on the socioeconomic status and the living condition. In the developing countries, EBV is usually acquired in early childhood (11, 12), probably through familial contact (13, 14). Improvement of economic and sanitary conditions might therefore explain the observed decrease in the rates of EBV-seroprevalence among children 5-9 years of age in urban Japan from more than 80% in 1990 to 59% between 1995 and 1999.

Primary EBV infections in young children are primarily manifested as nonspecific illness (15), and much less frequently as symptomatic IM (16). On the other hand, EBV infection in early childhood has become less common in developed countries, where 30 to 75% of college freshmen are seronegative for EBV (17). Primary EBV infections during or after the second decade of life is symptomatic in more than 70% of cases (17) and present as infectious mononucleosis (also known as kissing disease), which overall affects around 10% of the population in Europe and the US (10). Notably, the incidence of severe IM in adults seems to have increased over the last 20 years (18).

3.1.3. EBV biology and life cycle

EBV, also known as human herpesvirus 4 (HHV4) is a human DNA virus and a member of the family *Herpesviridae*, belongs to the subfamily *Gammaherpesvirinae*, genus *Lymphocryptovirus* (LCV; also named gamma-1 herpesviruses). LCV are found only in primates and EBV is the only LCV restricted to humans. The EBV genome consists of a linear double-stranded DNA molecule of around 184 kb in length. When a virus infects a cell, its linearized genome does not integrate into cellular DNA, but circularizes into an episome. The episome replicates after infection, leading to accumulation of multiple copies in the nucleus, which will be equally partitioned to daughter cells during future cell division (reviewed in (9)).

EBV is transmitted to new hosts via saliva containing infectious viral particles. EBV primarily infects the B cell latently *in vivo* as wells as *in vitro*, and to a lesser extent some type of epithelial cells. EBV entry in B cells is first mediated by attachment to the surface receptors CD21 (19) and CD35 (20), followed by binding to the HLA class II molecule, which will initiate the fusion of the enveloped virus with the membrane after endocytosis (21).

EBV, like all herpesviruses, displays two infection modes, i.e. latent and lytic infection (Fig. 1), which are characterized by different gene expression and are initiated by the transcription of specific viral trans-activators (the latent gene EBNA2 vs. the immediate-early lytic genes BZLF1 and BRLF1).

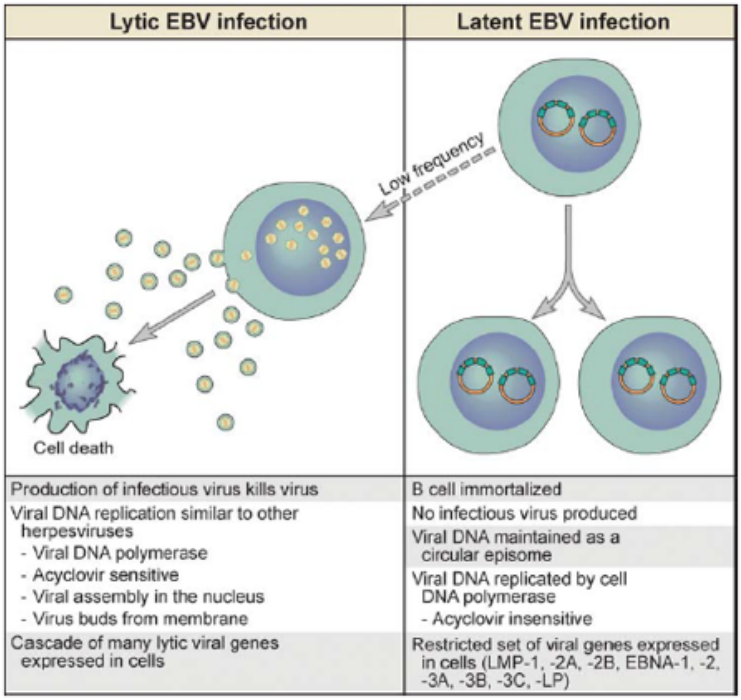


Figure 1: EBV life cycle (adapted from (9))

Latent EBV infection

EBV infection of primary B cells *in vitro* results in the expression of 8 latent viral proteins (6 nuclear proteins and 2 membranes proteins; table 2), leading to B cell transformation and the generation of lymphoblastoid cell lines (LCLs). These LCLs resemble proliferating B cell blasts with an activated phenotype. Moreover, latent antigens are detected in EBV-associated malignancies and several types of latency gene expression patterns can be found in these tumors (table 2). *In vivo*, EBV establishes a life-long latency exclusively in the memory B cell pool and up to 1 in 10^6 peripheral blood B cells are latently infected with the virus in healthy EBV carriers (22). During acute infectious mononucleosis (IM), the primary symptomatic EBV infection, up to half of all peripheral memory B cells carry EBV (23); however these cells are resting and do not express any viral antigens (24), whereas infected B cells in IM tonsils exhibit the complete set of latency gene expression (25). EBV latency genes have been

proposed to drive the differentiation of infected naïve B cells into long-lived memory B cells (26) and some of these genes are essential for B cell transformation *in vitro* (27).

Table 2. EBV latent antigens

EBV antigen	Required for immortalisation	Function known/postulated	Expressed in vivo in							
			PBM	IM	BL	NPC	HD	BLPD	TCL	Gastric carcinoma
EBNA1	+	Genome maintenance	?	+	+	+	+	+	+	+
EBNA2	+	Viral oncogene, transactivates cellular and other latent viral genes	–	+	–	–	–	+	–	–
EBNA3A	+	Activates cellular genes	–	+	–	–	–	+	–	–
EBNA3B	–	Activates cellular genes	–	+	–	–	–	+	–	–
EBNA3C	+	Viral oncogene, increases LMP1 expression	–	+	–	–	–	+	–	–
EBNA LP	+/–	Co-activates EBNA2 responsive genes, increases efficiency of immortalisation	–	+	–	–	–	+	–	–
LMP1	+	Viral oncogene, induces B cell activation and adhesion, protects from apoptosis	–	+	–	(+)	+	+	(+)	?
LMP2	–	Repression of lytic cycle, enhances B cell survival	+	+	–	+	+	+	+	+

(+)=expressed in a proportion of cases; PBM=peripheral blood mononuclear cells; HD=Hodgkin's lymphoma; EBNA=EBV nuclear antigen; IM=infectious mononucleosis; BLPD=B cell lymphoproliferative disease; LP=leader protein; BL=Burkitt's lymphoma; TCL=T cell lymphoma; LMP=latent membrane protein; NPC=nasopharyngeal carcinoma; ?=not known

Table 2: EBV latent genes (adapted from (28))

Lytic EBV infection

Lifelong latency in the host is a characteristic feature of herpesviruses such as EBV, cytomegalovirus (CMV) and herpes simplex virus (HSV). However, in order to outlive its host, a virus must transmit its genome to new hosts. Therefore, herpesviruses use periodic lytic reactivation in a fraction of latently infected cells to allow production of free infectious virions. EBV reactivation can be observed in the tonsils from IM patients (29, 30) and leads to release of infectious viral particles in the saliva (31). Triggering of the EBV lytic cycle *in vivo* is thought to occur in latently infected memory B cells differentiating into plasma cells (32). Lytic EBV replication proceeds through a sequential expression of viral gene categories (Fig. 2), starting with the expression of the two immediate early (IA) genes, followed by expression of the early viral genes, and finally of the late viral genes. The cascade begins when expression of the IE genes is triggered by host cell transcription factors, as observed upon cross-linking of B cell receptor with anti-IgG in EBV-positive Burkitt lymphoma cells *in vitro* (33). This process ends up with production and release of encapsidated, enveloped virions and ultimately with the lysis of the infected B cell.

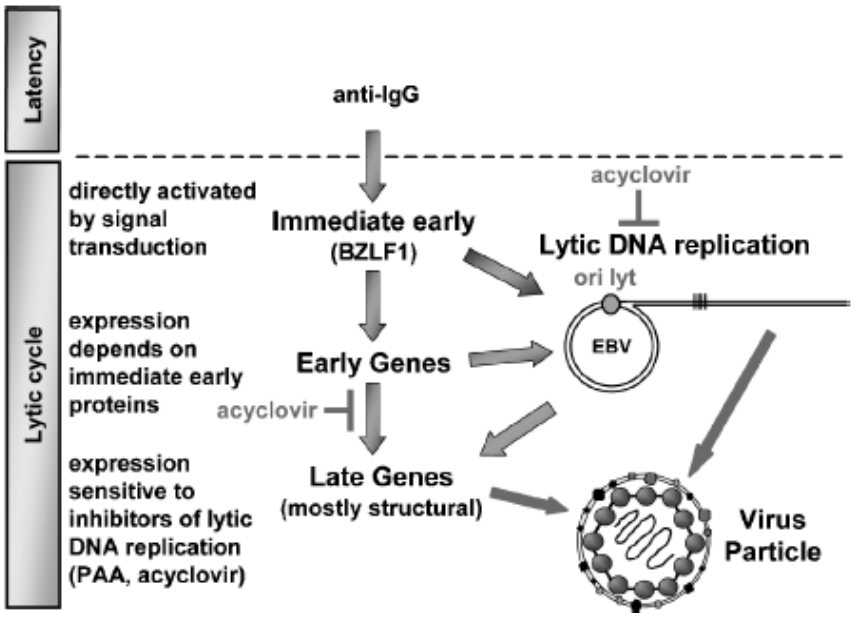


Figure 2. EBV lytic reactivation model upon anti-IgG treatment of EBV-positive Burkitt lymphoma cells (adapted from (34))

3.1.4. Infectious mononucleosis

Most primary EBV infections occur during early childhood and are either asymptomatic or result in non-specific symptoms. However, individuals that get infected after the age of 5 years bear the risk of developing infectious mononucleosis (IM) or kissing disease (10). This self-limiting disease is characterized by fever, tonsillitis, generalized lymphadenopathies, hepatosplenomegaly and fatigue (Fig. 3). Exaggerated IM-associated T cell responses, but not EBV infected B cells, are thought to cause the symptoms (35).

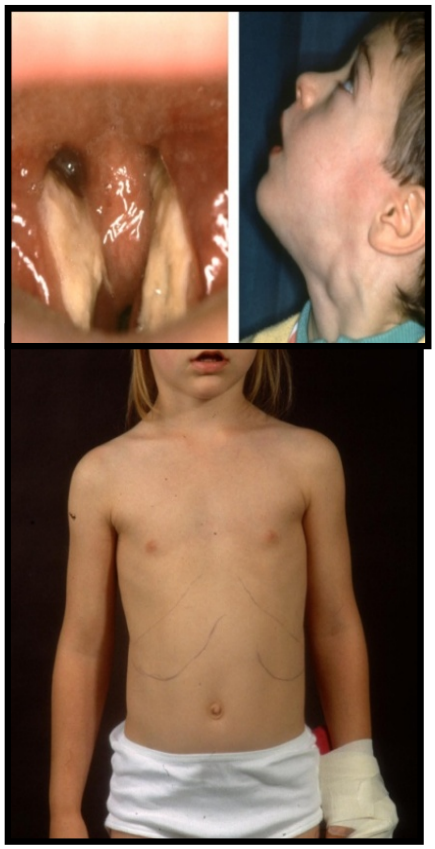
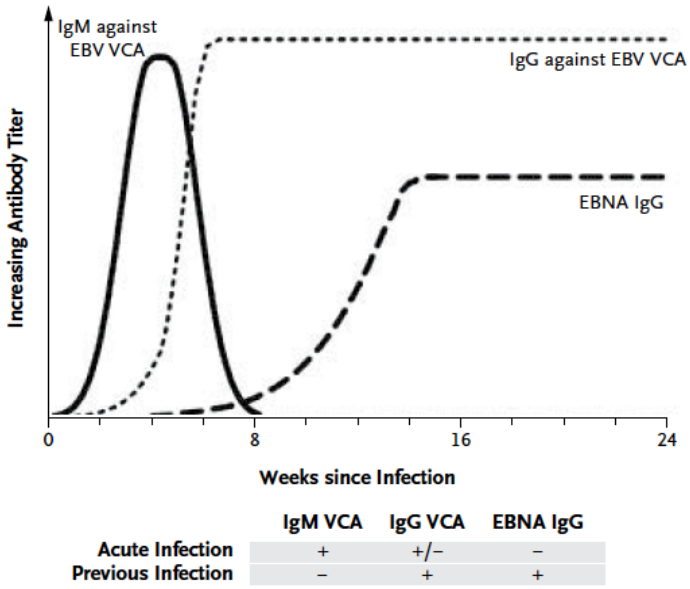


Figure 3. Clinical picture of infectious mononucleosis in a child, depicting tonsillitis, cervical lymphadenopathies and hepatosplenomegaly. (kindly provided by Prof. David Nadal, University Children's Hospital of Zurich)

While most patients with IM fully recover in two to four weeks, some patients experience persistent malaise and fatigue that can last several months.

The diagnosis of IM is usually based on the aforementioned characteristic clinical symptoms, the marked lymphocytosis, the presence of atypical lymphocytes (representing activated T cells) on peripheral-blood smear and a specific serological pattern. Most of patients with EBV-associated IM exhibit circulating heterophile IgM antibodies directed against viral antigens that cross-react with antigens found on sheep and horse red cells. Young children with IM might have a false negative heterophile test and need to be further tested for EBV-specific IgM and IgG serologies.



	IgM VCA	IgG VCA	EBNA IgG
Acute Infection	+	+/−	−
Previous Infection	−	+	+

Figure 4: Antibody responses during primary EBV infection (adapted from (10))

Acute primary EBV infection is confirmed by the presence of IgM to the viral capsid antigen (VCA) and the absence of IgG to EBNA (Fig. 4). Heterophile-negative IM might also be observed in acute cytomegalovirus (CMV), human immunodeficiency virus (HIV) and toxoplasmosis infections.

3.1.5. Innate immune responses to EBV

Innate immunity to EBV is mainly mediated by pathogen-associated molecular pattern receptors such as Toll-like receptors (TLRs), different subsets of dendritic cells (DCs) and natural killer (NK) cells and this topic has been extensively reviewed in the attached review (manuscript N°2 (36)).

3.1.6. T cell responses to EBV

Acute IM is associated with an expansion of activated cytotoxic $CD8^+$ T cells, but not of $CD4^+$ T cells (37), which are oligoclonal (38) in their T cell receptor (TCR) usage (39). Moreover, these proliferating $CD8^+$ T cells (Fig. 5) were shown to be EBV-specific using HLA class I tetramer (40) and *ex vivo* cytotoxic assays (41), and to be mostly directed against epitopes derived from immediate early and early lytic antigens (42). The dynamics of T cell responses parallels the rapid decrease of EBV-positive cells in peripheral blood during acute IM (23). On the other hand, IM patients exhibit prolonged oral EBV shedding up to one year after IM (31, 43). Persistent EBV infection in healthy EBV carriers is primarily controlled by T cells (35). Moreover, clinical evidence from transplant patients strongly suggests that latent antigen-specific $CD8^+$ T cells are major mediators of host control *in vivo* (44). Immunosuppressive treatment or co-infection with HIV or *Plasmodium falciparum* in holoendemic malaria regions compromises this EBV specific T cell immunity and leads to increased frequencies of EBV-associated malignancies like post-transplant lymphoproliferative disease and Burkitt lymphoma (9). Accordingly, some of these EBV-associated malignancies can be treated by adoptive transfer of EBV antigen-specific, *in vitro* expanded T cells (45).

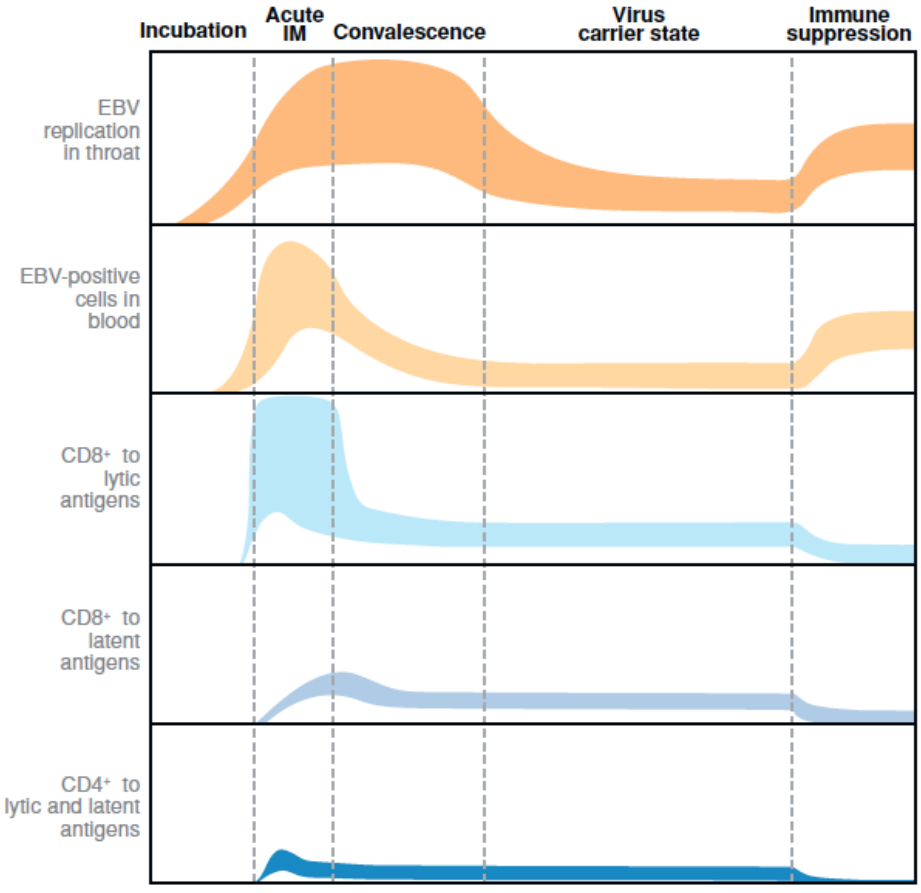


Figure 5: Peripheral blood EBV-specific T cell responses and viral loads in saliva and peripheral blood during the course of an infectious mononucleosis (from (35))

Therefore, the priming and maintenance of this EBV specific T cell immune control is essential for asymptomatic carriage of this oncogenic virus, and the conditions under which this occurs need to be characterized to identify individuals that are susceptible to EBV-associated malignancies and to mimic these conditions for successful vaccination.

3.1.7. Hypothesis for infectious mononucleosis

Along these lines it has become clear that symptomatic primary EBV infection manifested as IM does not efficiently establish EBV immune control and therefore bears an increased risk for developing EBV-associated diseases like classical Hodgkin lymphoma (46) and multiple sclerosis (47). Nearly 45 years after the discovery of the link between EBV and IM (3), the mechanisms leading to this frequent disease in only some of EBV infected individuals still remain unknown.

It has been proposed that asymptomatic primary EBV infection is associated with early adaptive T cell control of EBV in the tonsils (48), without major changes of the peripheral blood T cell compartment (49). It has been further postulated that higher viral doses lead to stronger cytotoxic T cell (CTL) responses and therefore to IM symptoms. Indeed, the dose of orally acquired EBV might be larger during deep kissing in adolescents compared to oral transmission during early childhood (50). Another explanation might be found in the heterologous immunity concept (51), in which infection during early childhood elicits powerful naive CTL responses that rapidly contain the infection. However, in older individuals, the T-cell memory compartment, which contains numerous pathogen-specific memory T cells, is more likely to elicit weak cross-reactive heterologous CTL response that clears the virus inefficiently. Thus, to eliminate infection, cross-reactive T cells, which have been observed in IM (52), must be activated more extensively, resulting in the massive CTL expansion in IM. Finally, others have proposed that specific polymorphisms of genes such as the anti-inflammatory cytokine IL-10 (53) or MHC class I (54) might influence the immune control of EBV and therefore predispose for IM.

We postulated that IM is linked to inefficient innate NK cell-mediated immunity to the virus, allowing EBV to replicate to high titers initially (55). This hypothesis will be elaborated in the subject of investigation and the discussion parts of the thesis.

3.2. Natural killer cells

3.2.1. NK cell functions and biology

NK cells were originally described as large granular lymphocytes with natural cytotoxicity against tumor cells (56-58). This cytotoxic ability did not require any priming and was not restricted by the target cell's expression of major histocompatibility complex (MHC) molecules. Experiments in mouse models of bone marrow graft rejection (59) led to the proposal that NK cells would kill any target that lacked self–major histocompatibility complex (MHC) class I molecules (the "missing self" hypothesis) (60). NK cells were later recognized as a separate lymphocyte lineage, with both cytotoxic and cytokine-producing effector functions (Fig. 7), and were defined as $CD3^-$ $CD56^+$ lymphocytes (61) in humans. Their antiviral role was first confirmed in mice infected with murine cytomegalovirus (MCMV) using NK cell depletion and adoptive transfer experiments (62).

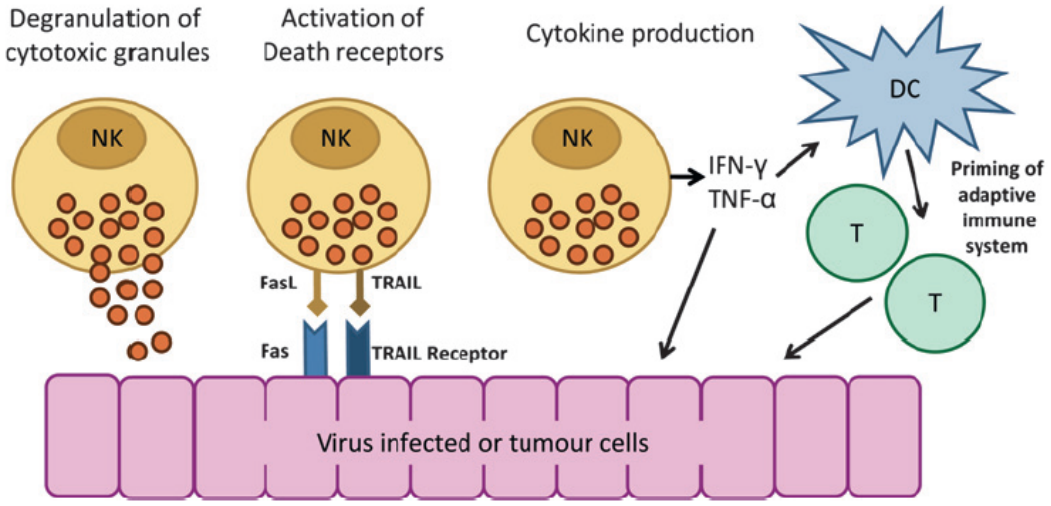


Figure 7: NK cell effector functions (adapted from (63)). NK cells can kill target cells via directed degranulation of cytotoxic granules or via engagement of death receptors. NK cells also secrete large amounts of proinflammatory cytokines such as IFN-γ, which will act on virus infected cells or modulate the functions of other immune cells such as dendritic cells (DCs).

Recently, NK cells have also been shown to shape the adaptive immune response (reviewed in (64)), either by acting on antigen presenting cells (APCs), or by direct killing of activated T

cells (Fig. 8). Finally, NK cells seem to play a role in reproduction (65) and in hematopoietic stem cell transplantation (66) and have been recently classified as members of the group 1 innate lymphoid cells (ILCs) (67)

NK cells can kill virus-infected cells or transformed cells via secretion of cytotoxic granules containing perforin and granzyme B, and can secrete large amount of proinflammatory cytokines such as IFN-γ. These immune cells recognize target cells via germline-encoded surface receptors, that unlike their counterparts on T or B cells, do not require somatic recombination and are therefore present at higher precursor frequencies in the NK cell compartment, ready to raise rapid and substantial immune responses after activation (68). Despite having been originally described as "natural killer", NK cells from mouse and human require priming by various factors, such as IL-15 presented by DC (69) or macrophages (70), IL-12 (71) or IL-18 (72), to achieve their full effector potential, highlighting the regulatory interactions between NK cells and other immune cells.

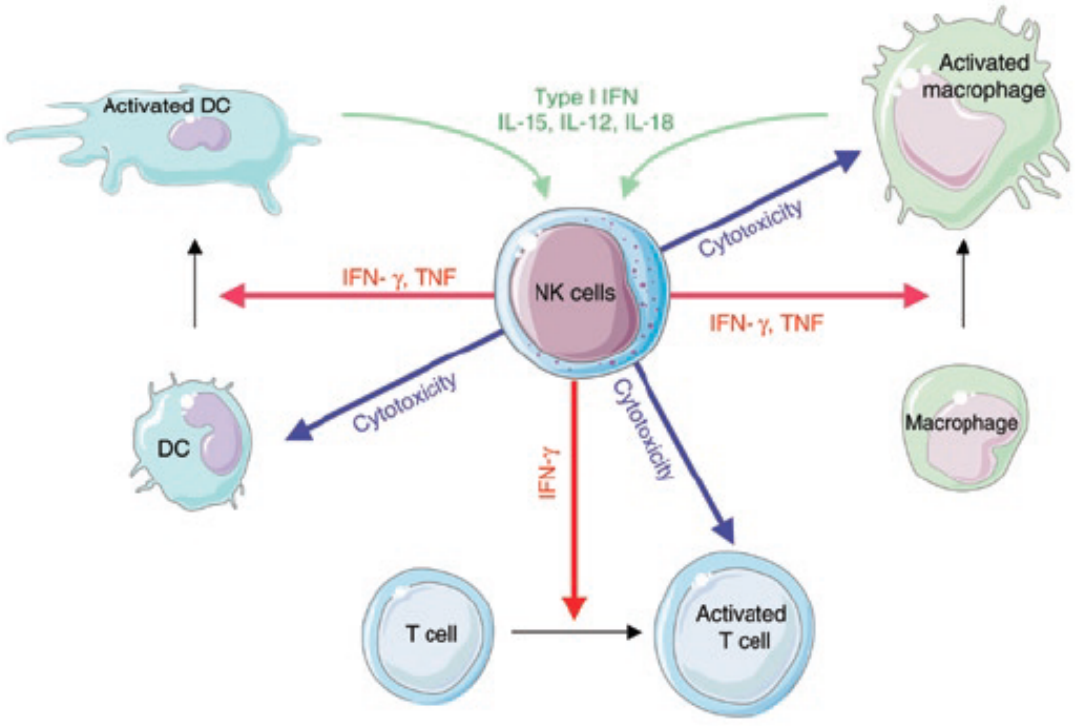


Figure 8: Regulations of adaptive immune responses by NK cells (adapted from (73))

The activation of NK cell cytotoxicity is mediated by a balance of inhibitory and activating NK cell receptors (Fig. 9), as well as various adhesion and co-stimulatory molecules (74, 75).

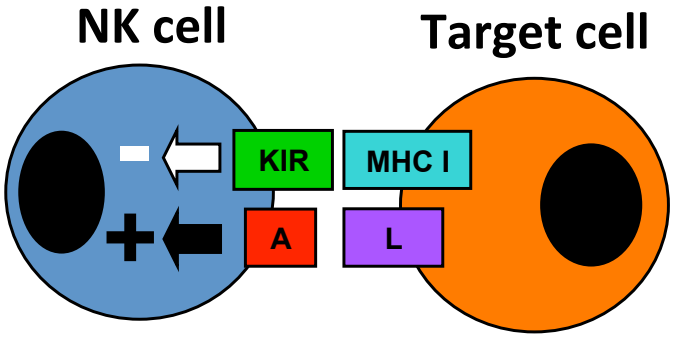


Figure 9: Simplified model of target cell recognition by NK cells. Activating ligands (L; violet) are upregulated on target cells and trigger activating receptors (A; red), which deliver activating signals. Inhibitory ligands such as the MHC class I (blue) on the surface of the target cell engage inhibitory NK cell receptors such as the killer immunoglobulin-like receptors (KIRs) (green) and provide inhibitory signals. The balance of signals will ultimately determine the NK cell response, i.e. tolerance or cytotoxic degranulation.

Inhibitory receptors and NK cell licensing

Under normal circumstances of immune surveillance of self, inhibitory NK cell receptors recognize MHC class I molecules as their cognate ligands present on virtually every cell in the body. Notably, virus-infected cells or transformed cells can downregulate the expression of MHC class I in order to avoid recognition by cytotoxic T cells. Inhibitory human NK cell receptors include the killer immunoglobulin-like receptors (KIRs) that bind to classical MHC class Ia ligands HLA-A, -B and-C (76, 77), and the inhibitory CD94-NKG2A heterodimeric C-type lectin-like receptors that bind the non-classical MHC class Ib HLA-E (78). These inhibitory receptors are expressed in a variegated fashion, so that each NK cell expresses multiple receptors in a complex combinatorial pattern of inhibitory receptor repertoire (79, 80). These inhibitory signals are thought to be essential to avoid NK cell mediated killing of healthy self cells, i.e. to prevent autoimmunity. Notably, the interactions between inhibitory receptors, including NKG2A (81), and their MHC class I ligands during NK cell maturation allow the licensing (or education) of the NK cell, which become fully functional (82). Failure to receive the licensing signal keeps NK cells in a hypo-responsive state (83).

Activating receptors

For many years, NK cells were thought to be controlled only by inhibitory signals, with killing of target cells resulting from the absence of inhibition. However, it is now well established that NK cells require specific activating signals – and even a combination thereof (84) - leading to cytotoxicity. Upregulation of activating NK cell ligands have been described on virus-infected cells, tumor cells and during cellular stress, e.g. during activation of the DNA damage response (85). In humans, major triggering NK cell receptors include NKG2D (receptor for the stress-associated MICA/B and ULBP molecules), DNAM1 (receptor for CD112 and CD155), 2B4 (receptor for CD48), activating KIRs (receptors for HLA class I molecules), NKG2C (receptor for HLA-E), the natural cytotoxic receptors (NCRs) NKp46, NKp44 and NKp30, and CD16 (or FcγRIIIa; low affinity IgG receptor for antibody-dependent cellular cytotoxicity).

3.2.2. NK cell subsets and differentiation

NK cells are, along with T and B cells, members of the lymphocyte lineage, which derive from common lymphoid progenitors found in the bone marrow. The first differentiation stage – the pro-NK cell - migrates from the bone marrow to secondary lymphoid tissues (SLT) such as lymph nodes and tonsils. Freud et al. described five stages of NK cell maturation in SLT (86), which can be defined by the expression of surface markers (Fig. 10).

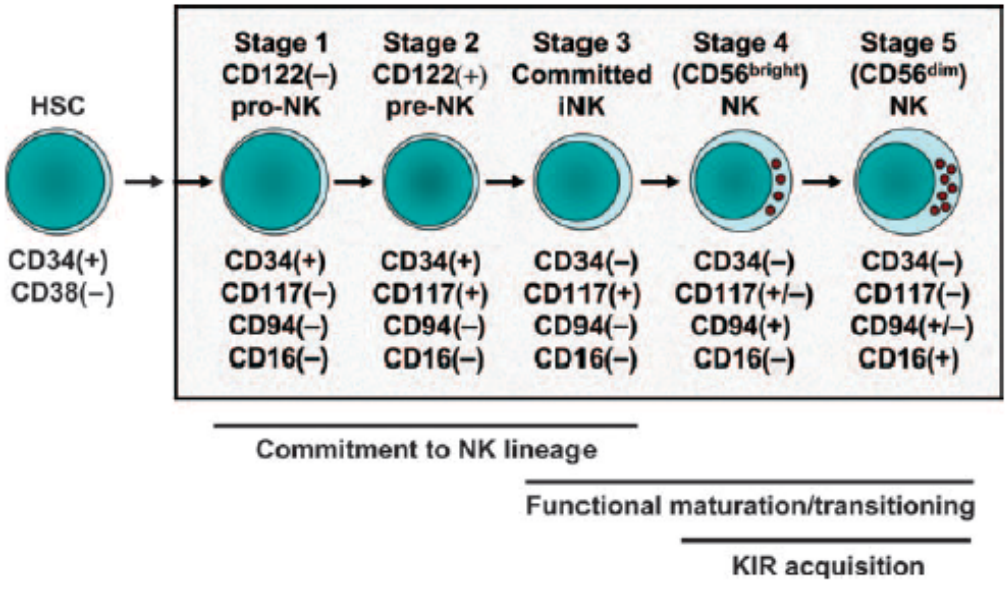


Figure 10: Proposed model of NK cell differentiation in SLT (adapted from (87))

The pro-NK cell, pre-NK cell, iNK cell and CD56^{bright} NK cell populations are enriched within SLT. In this model, the CD56^{dim} NK cells, which are enriched in the peripheral blood (88, 89), constitute the final stage of NK cell differentiation, which is completed outside of the SLT. The acquisition of the inhibitory receptor CD94/NKG2A in the CD56^{bright} NK cell stage in SLT is important because this developmental stage begins to acquire cytotoxicity, including perforin-mediated and Fas ligand-mediated killing, and inflammatory cytokine production (90). There is reasonable evidence that CD56^{bright} NK cells further develop into the CD56^{dim} subset (91-93), but the factors driving this process remain unknown.

The blood NK cell compartment is mainly composed of the two well-characterized functional CD56^{bright} CD16^{-} and CD56^{dim} CD16^{+} subsets (94). The former NK cell subset produces large amounts of cytokines upon monokine stimulation, acquires cytotoxicity only after prolonged activation and is enriched in secondary lymphoid organs (88). The latter NK cell subset readily kills susceptible targets and can rapidly secrete IFN-γ upon engagement of activating receptors (95).

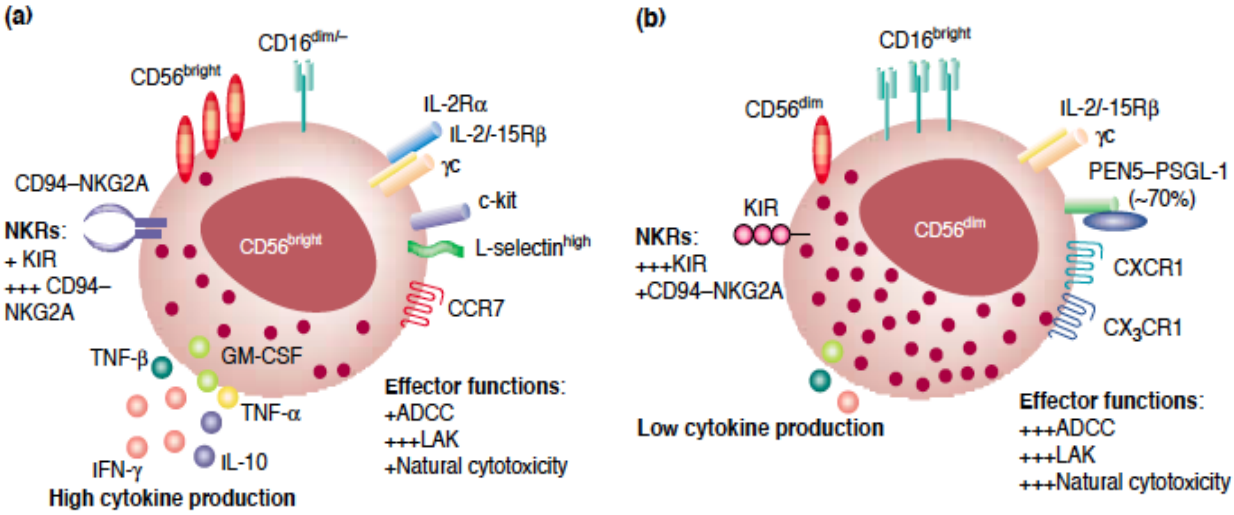


Figure 11: Phenotype and functions of CD56^{bright} CD16^{-} (a) and CD56^{dim} CD16^{+} (b) NK cells (adapted from (96))

Moreover, both NK cell subsets significantly differ in expression of inhibitory receptors, chemokine receptors and cytokine receptors (Fig. 11). Indeed, CD56^{bright} CD16^{-} NK cells express NKG2A, but no KIRs, homing receptors for SLT (CD62L and CCR7) and the high-affinity IL-2 receptor (IL2Rαβγ). On the other hand, the CD56^{dim} CD16^{+} NK cells display the full set of inhibitory receptors, with variegated expression of NKG2A and one or multiple

KIR haplotypes (97). Moreover, Juelke et al. reported the presence in peripheral blood of CD56^{dim} CD62L^{+} NK cells, which exhibited intermediate phenotype and functionality, compared to CD56^{bright} and CD56^{dim} CD62L^{-} NK cells (98).

A growing body of evidence indicates that NK cell differentiation continues within the mature CD56^{dim} NK subset, as demonstrated by *in vitro* experiments and longitudinal studies of patients with acute viral infections. Indeed, discrete maturation stages of CD56^{dim} NK cells can be assessed by expression of the two types of inhibitory receptors NKG2A and KIRs (Fig. 12 and 13), spanning from the early-differentiated CD56^{dim} NKG2A^{+} KIR^{-} to the late differentiated CD56^{dim} NKG2A^{-} KIR^{+} (99, 100).

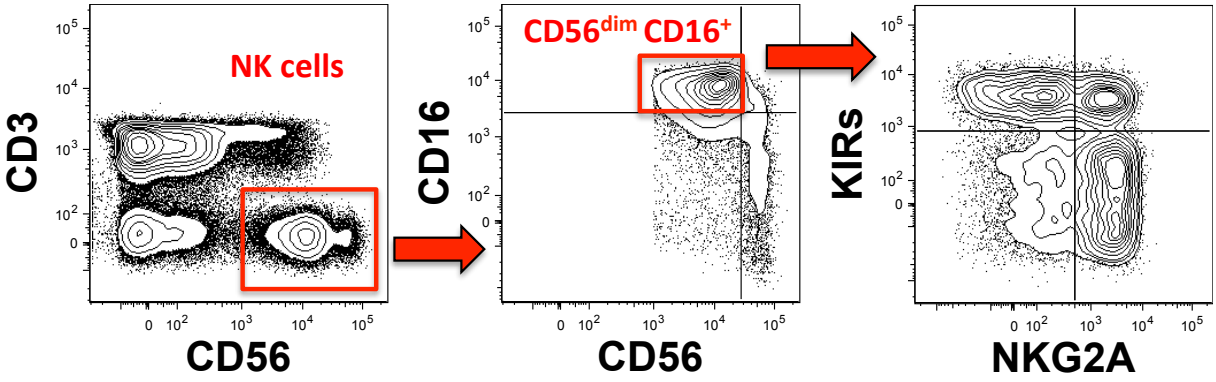


Figure 12: Gating strategy to assess CD56^{dim} differentiation stages in peripheral blood by flow cytometry.

Recently, CD57 has been proposed as a marker of terminal differentiation of CD56^{dim} NK cells (100, 101) and has been shown to be up-regulated on proliferating CD56^{dim} NKG2C^{+} NK cells after acute infection with CMV (102), hantavirus (103) and chikungunya virus (104). Terminally differentiated CD57^{+} CD56^{dim} NK cells exhibit increased cytotoxicity, but decreased proliferative capacity and responsiveness to cytokines *in vitro*.

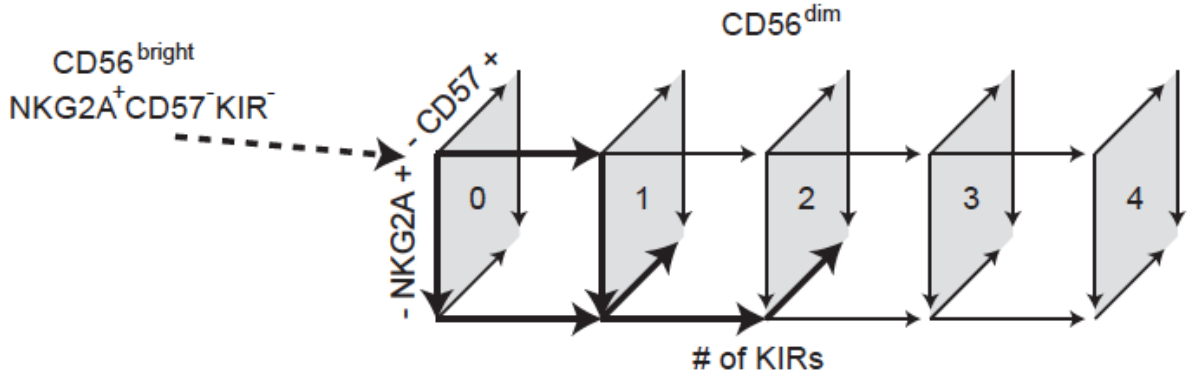


Figure 13: Proposed model of NK cell differentiation in peripheral blood (adapted from (100)). Differentiation of $CD56^{dim}$ NK cells is hallmarked by sequential, but not linear, loss of NKG2A and acquisition of single or multiple KIRs, as well as up-regulation of CD57.

3.2.3. Antiviral role of NK cells

The protective role of NK cells against several types of viruses in mouse models is well accepted and has been extensively characterized mainly in mouse cytomegalovirus (MCMV) infection (105). From this model, we know that NK cells play a non-redundant role, which is exclusively conferred upon a well-defined NK cell subset bearing the Ly49H receptor. This activating receptor was shown to directly bind to the viral protein (m157) expressed on the surface of virus-infected cells.

Recently, several groups reported the generation of memory-like NK cell responses during allergic reactions (106) and viral infections (107, 108) in experimental mice. In the MCMV model, $Ly49H^{+}$ NK cells exhibit adaptive-like immune responses similar to those described during antigen-specific T cell responses. Indeed, this NK cell subset exhibits over the course of MCMV infection the five phases observed during memory immune responses, namely clonal expansion, contraction, persistence of functional memory cells in tissues and finally recall or enhanced secondary responses upon viral re-challenge. NK cells combine, thus, functions that are attributes of both innate and adaptive immunity, blurring the borders between these two arms of the immune response (109).

In humans, the role of NK cells in viral infections is less clear because of the extreme rarity of patients with isolated NK cell deficiencies. Nevertheless, these patients, which possess normal T cell responses, exhibit an increased susceptibility to viral infections, mainly to herpesviruses (110). This suggests a potential non-redundant function of NK cells, which obviously cannot be confirmed in immunocompetent individuals. However, new models of mice with human immune system components have been proven useful to study the early innate human NK cell responses during infections with human viruses such as EBV (111, 112).

Recent longitudinal studies of patients with acute viral infections have reported the preferential expansion of a defined NK cell subset in peripheral blood. Indeed, acute cytomegalovirus (CMV) and hantavirus virus infections elicit the persistent accumulation of the late-differentiated $CD56^{dim}$ $NKG2C^{+}$ NK cell subset. Interestingly, $CD56^{dim}$ $NKG2C^{+}$ NK cells are found at increased counts in CMV-positive individuals (113) and preferentially

proliferate upon exposure of CMV-infected fibroblasts to PBMCs *in vitro* (114). Nevertheless, none of these studies could show that the expanding NK cell subset can directly target CMV- or hantavirus-infected cells. Therefore, it remains unclear if such expansions of NK cell subsets might be protective or just a bystander inflammatory response with potential detrimental effects (115).

Several lines of evidence indicate a potential role of NK cells in EBV infection. Firstly, overall NK cell numbers expand during IM (17, 116-119); secondly, a subset of NK cells, which is enriched in the tonsils, restricts the EBV-driven transformation of B cells (120); thirdly, NK cells can kill lytically-replicating B cells *in vitro* (121); fourthly, a recent report on XMEN patients which suffer from chronic EBV infection, Mg^{2+} deficiency and neoplasia, highlighted the non-redundant role of the activating NK cell receptor NKG2D in the control of this tumor-associated virus (122); and finally, NK cells exhibit a protective role in humanized mice infected with EBV (123). Nevertheless, the contribution of particular NK cell subsets to the immune control of EBV, especially during primary infection, remains elusive.

4. SUBJECT OF INVESTIGATION

We postulated that inefficient innate immune control mediated by NK cells allows EBV to initially replicate to higher viral loads leading to stronger stimulation and expansion of the T cell compartment as observed in IM (124). Moreover, this NK cell-mediated immune control might be optimal during early childhood, but impaired in older individuals. Indeed, NK cells from children younger than 5 years might better restrict EBV during the early phase of infection and therefore decrease the number of EBV infected B cells compared to teenagers and adults. This decreased antigenic load will ultimately elicit less T cell stimulation during the adaptive immune response (Fig. 14).

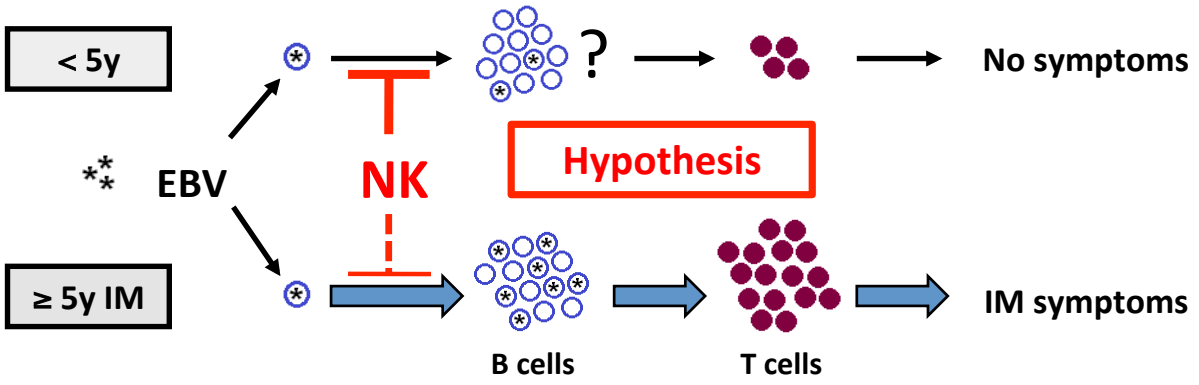


Figure 14: Hypothesis for IM. Age-dependent NK cell deficiency in EBV immune control predispose for infectious mononucleosis. Impaired NK cell-mediated early immune control of EBV in individuals older than 5 years leads to increased numbers of EBV-infected B cells, which will elicit the extensive T cells responses associated with IM symptoms.

The subject of this thesis was to investigate the NK cell response during acute primary symptomatic EBV infection or infectious mononucleosis (IM), the specific NK cell-mediated recognition of both EBV life forms (lytic vs. latent) and to assess possible age-related changes in the phenotype or function of NK cells that could influence the early immune control of EBV and therefore the course of primary infection. We also aimed to better understand the

NK cell-mediated immune control of EBV in the tonsils and to assess which factor might influence the balance between EBV lytic and latent cycles in this secondary lymphoid organ.

To address this subject, the following topics were investigated:

1) Do possible age-related changes in the phenotype and function of NK cell alter the recognition of EBV-infected B cells at different ages of life and therefore influence the course of primary EBV infection?

Primary EBV infections before 5 years of age often run an asymptomatic course. Older individuals have however an increased risk of developing symptomatic primary infection, i.e. infectious mononucleosis, which is associated with an increased risk to develop autoimmune diseases and EBV-positive tumors. Age-related differences in immune responses have been described, but such differences have not been assessed in the context of NK cell-mediated immune control of EBV. Thus, we investigated the age-related changes in the distribution of blood NK cell subsets, in the expression of different sets of NK cell receptors and in the *in vitro* responses of NK cells against EBV-infected B cells.

2) Does a particular NK cell subset expand and accumulate in peripheral blood of patients with acute IM, and if yes, which EBV infection programs does this NK cell subset specifically target?

Several reports of patients with acute viral infections have described the accumulation of a specific NK cell subset in peripheral blood, but none of these studies could demonstrate a direct recognition of the infected cells by the expanding NK cell subset. The role of NK cells in the immune control of EBV during primary infection remains largely unknown. Thus, we longitudinally studied the NK cell responses during infectious mononucleosis and comprehensively assessed the accumulation, maturation and proliferation of each NK cell subset over the course of primary infection. Furthermore, we tested their reactivity against infected B cells with latent or lytic EBV infection *in vitro* and their ability to proliferate in an *in vitro* model of primary EBV infection.

5. RESULTS

NK cell cytotoxic degranulation against EBV transformed B cells does not differ with age

Primary EBV infection is asymptomatic in children < 5 years of age, but it may manifest as infectious mononucleosis in older individuals. The reason for this age-dependent predisposition to IM is unknown. We postulated that NK cell-mediated early immune control of EBV is impaired in individuals > 5 years of age compared to younger children. A decreased NK cell-mediated immune control might be caused firstly by a decreased frequency of a functional NK cell subset within the bulk NK cell population, which might be protective during EBV infection; secondly, this might be explained by an impaired NK cell-mediated recognition of EBV-infected cells with either latent or lytic EBV replication or both; thirdly, since some activating receptors have been shown to be involved in the NK cell-mediated recognition of EBV-transformed B cells and or EBV-infected B cells with lytic replication, an age-dependent decrease in the surface expression of such receptors might lead to decreased NK cell responses.

We first compared the frequencies of the two main NK cell subsets found in the peripheral blood, $CD56^{bright}$ $CD16^{-}$ and the $CD56^{dim}$ $CD16^{+}$, in healthy uninfected individuals with different ages, from the newborn to the young adult (Fig. 14). The former subset has been shown to restrict the EBV-driven transformation of B cells via secretion of IFN-γ (120). On the other hand, the latter subset might play a role in the killing of EBV-infected cells with lytic reactivation (121).

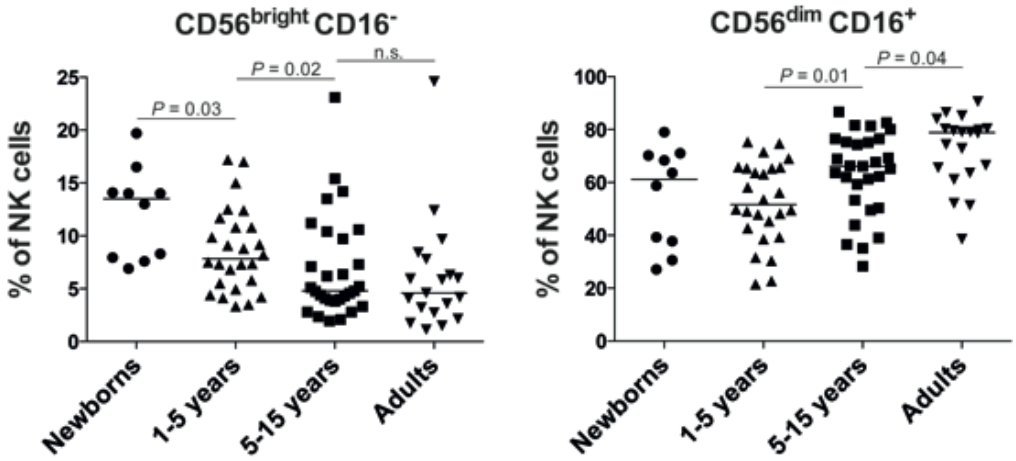


Figure 14: Frequencies of CD56^{bright} CD16^{-} and the CD56^{dim} CD16^{+} subsets in healthy individuals with different ages. PBMCs from newborns **(**cord blood mononuclear cells or CBMCs, n=10), from children aged 1-5 years (n=26), from children and teenagers 5-15 years (n=29) and from adults aged 20-30 years (n=19) were stained with monoclonal antibodies (mAb) and assessed by flow cytometry (see Fig. 12).

We observed a decrease in the frequencies of the cytokine-secreting CD56^{bright} CD16^{-} subset with increasing age and the opposite pattern for the CD56^{dim} CD16^{+} subset. This could indicate that younger children might better control the EBV transformation of B cells in peripheral blood during acute EBV infection. However, EBV gene expression associated with transformation has only been observed in the tonsils (25), and not in the peripheral blood during acute IM, and IM elicits the preferential accumulation in peripheral blood of a CD56^{dim} NK cell subset (see manuscript 1). Therefore, it might be interesting to assess the frequencies of these NK cell subsets in the tonsils from healthy individuals with different ages in future studies.

We next assessed the surface expression level of activating NK cell receptors involved in the recognition of EBV-infected cells *in vitro* on CD56^{dim} NK cells from individuals with different ages. Indeed, the expression level of such triggering receptors was shown to positively correlate with the magnitude of NK cell activation and cytotoxicity (125). NK cell recognition of lymphoblastoid cell lines (LCLs; latent EBV) is mainly mediated by 2B4 (receptor for CD48) and by LFA-1 (receptor for ICAM1), and both ligands are known to be up-regulated on transformed EBV-infected B cells (126, 127).

Additionally, NK cell-mediated killing of EBV-positive Burkitt lymphoma cells with lytic reactivation is impaired upon blocking of the NKG2D ligand ULBP1 and the DNAM1 ligand CD112, suggesting an important role of these triggering receptors in the control of EBV lytic replication (121).

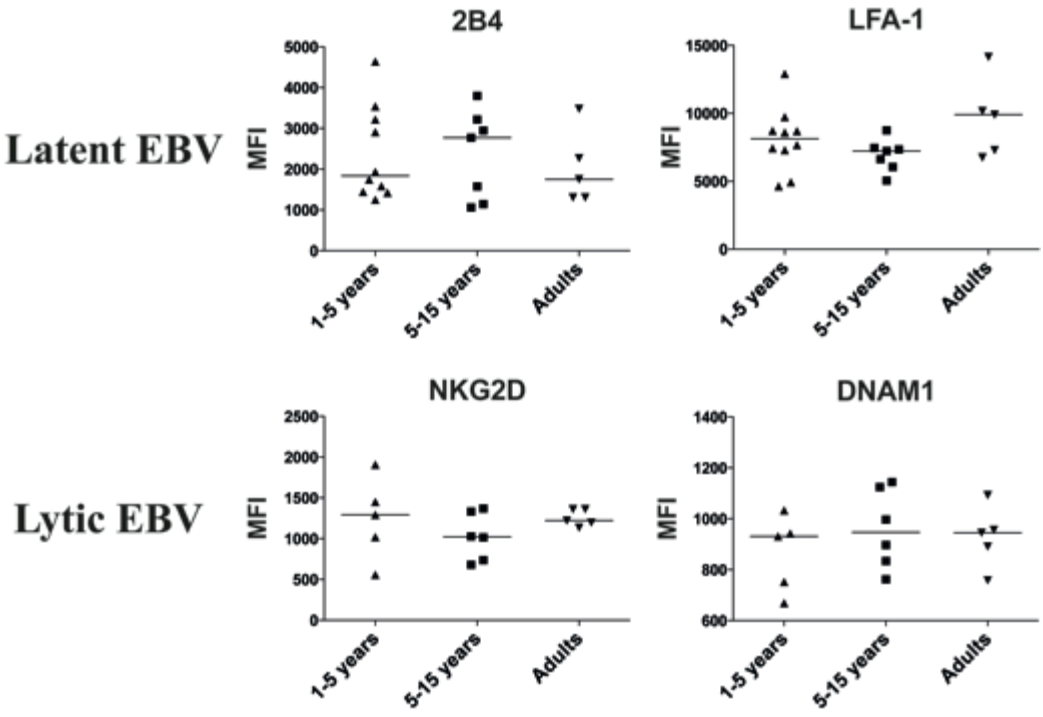


Figure 15: Surface expression level of activating NK cell receptors involved in recognition of latent EBV, i.e. 2B4 and LFA-1, and of lytic EBV, i.e. NKG2D and DNAM1 in healthy individuals with different ages. PBMCs from children aged < 5 years, children aged 5-15 years and adults aged 20-30 years were stained with mAb and analyzed by flow cytometry. The mean fluorescence intensities (MFI) of each receptor were quantified on $CD56^{dim}$ NK cells using FlowJo.

We observed no significant age-related changes in the expression of these triggering receptors (Fig. 15), suggesting that EBV-infected B cells expressing activating ligands might elicit a similar cytotoxicity from NK cells at different ages of life.

We finally investigated the *in vitro* responses of NK cell from individuals with different ages against LCLs. For this assay, we used the allogeneic MHC class I competent LCL 721.45 as target cells, which resembles autologous LCLs in terms of activating ligands, but differs in MHC class I haplotype expression (indicating possible mismatch KIRs-HLA class I). We included additionally the MHC class I deficient LCL 721.221 and the MHC class I deficient erythroleukemia cell line K562 as controls. We assessed the cytotoxic degranulation (CD107a mobilization) of $CD56^{dim}$ NK cells from healthy individuals at different age of life (Fig. 16).

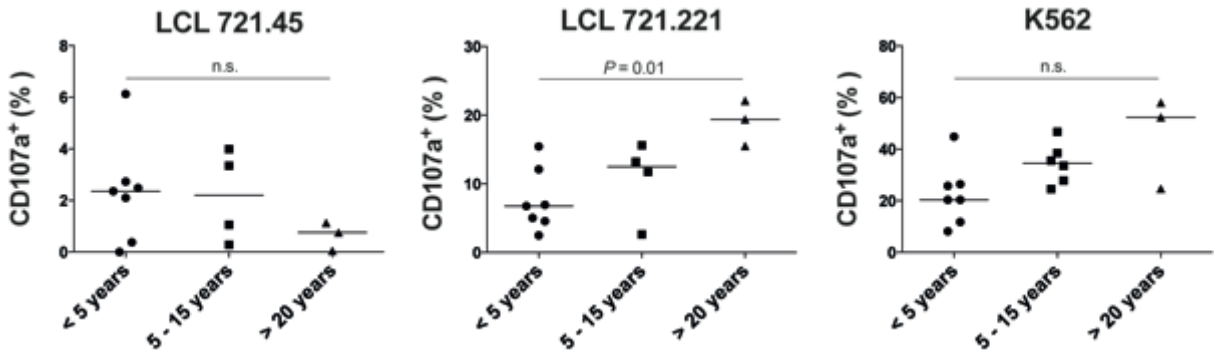


Figure 16: Cytotoxic degranulation of CD56^{dim} NK cells upon co-culture with different target cells. Freshly isolated PBMCs were stimulated overnight with 100 U/ml rIL-2 and were co-cultured the next day with LCL721.45 (MHC class I positive), LCL721.221 (MHC class I negative) or K562 (MHC class I negative) at an effector to target (E:T) ratio of 10:1 for 6 hours. Frequencies of degranulating CD107a^{+} cells within the CD56^{dim} NK cells were assessed by flow cytometry at the end of the co-culture.

The cytotoxic degranulation of CD56^{dim} NK cells against MHC class I deficient target cells (LCL721.221 and K562) increased by more than 2-fold from early childhood to adulthood. This pattern fits with the age-related increase in the frequency of CD56^{dim} KIRs^{+} (see manuscript 1), which might better target MHC class I deficient cells. We observed an overall low cytotoxic degranulation against the MHC class I competent LCL 721.45 and no significant changes with age. This low response is explained by the EBV-driven up-regulation of MHC class I (HLA A, B, C) and HLA-E molecules (Fig. 17) on EBV infected B cells (128), which deliver strong inhibitory signals to the NK cell.

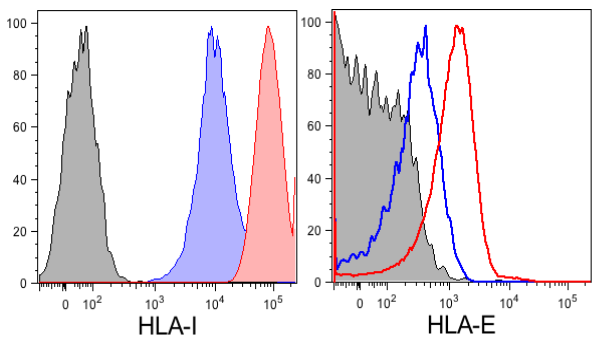


Figure 17: HLA class I and HLA-E expression levels on EBV-infected B cells (red histogram) and autologous uninfected B cells (blue histogram). LCLs from one donor were generated by infection with wildtype B95.8 EBV of primary B cells and cultured for one month. Autologous PBMCs were thawed and cultured overnight. The next day, LCLs and autologous PBMCs were stained with mAb and analyzed by flow cytometry. HLA class I and HLA-E expression were assessed on $CD19^+$ B cells using Flow Jo.

Altogether, we could not demonstrate any age-related changes in the NK cell reactivity to infected B cells with latent EBV (LCLs). It would be important to also test the age-related NK cell responses against infected B cells with lytic replication, since the switch from latent to lytic EBV replication has been shown to enhance the NK cell responses (121). This effect has been shown to be mediated by down-regulation of inhibitory surface ligands (MHC class I and HLA-E) and up-regulation of activating ligands (ULBP-1 and CD112) during EBV lytic replication (121). However, one would need to test these NK cell responses against target cells with EBV lytic replication in an autologous setting in order to avoid possible biases from KIR-HLA class I haplotype mismatches.

CLINICAL STUDY DESIGN

Recruitment of case patients and control subjects

Pediatric patients (age 1-16 years) presenting between October 2010 and April 2013 on the emergency department of the University Children's Hospital of Zurich with suspected IM were invited to participate in the study. Patients and parents were informed about the clinical study protocol orally by the responsible physician and in writing using a patient information sheet. Informed consent were obtained either from the patient himself depending on his age and from his parents.

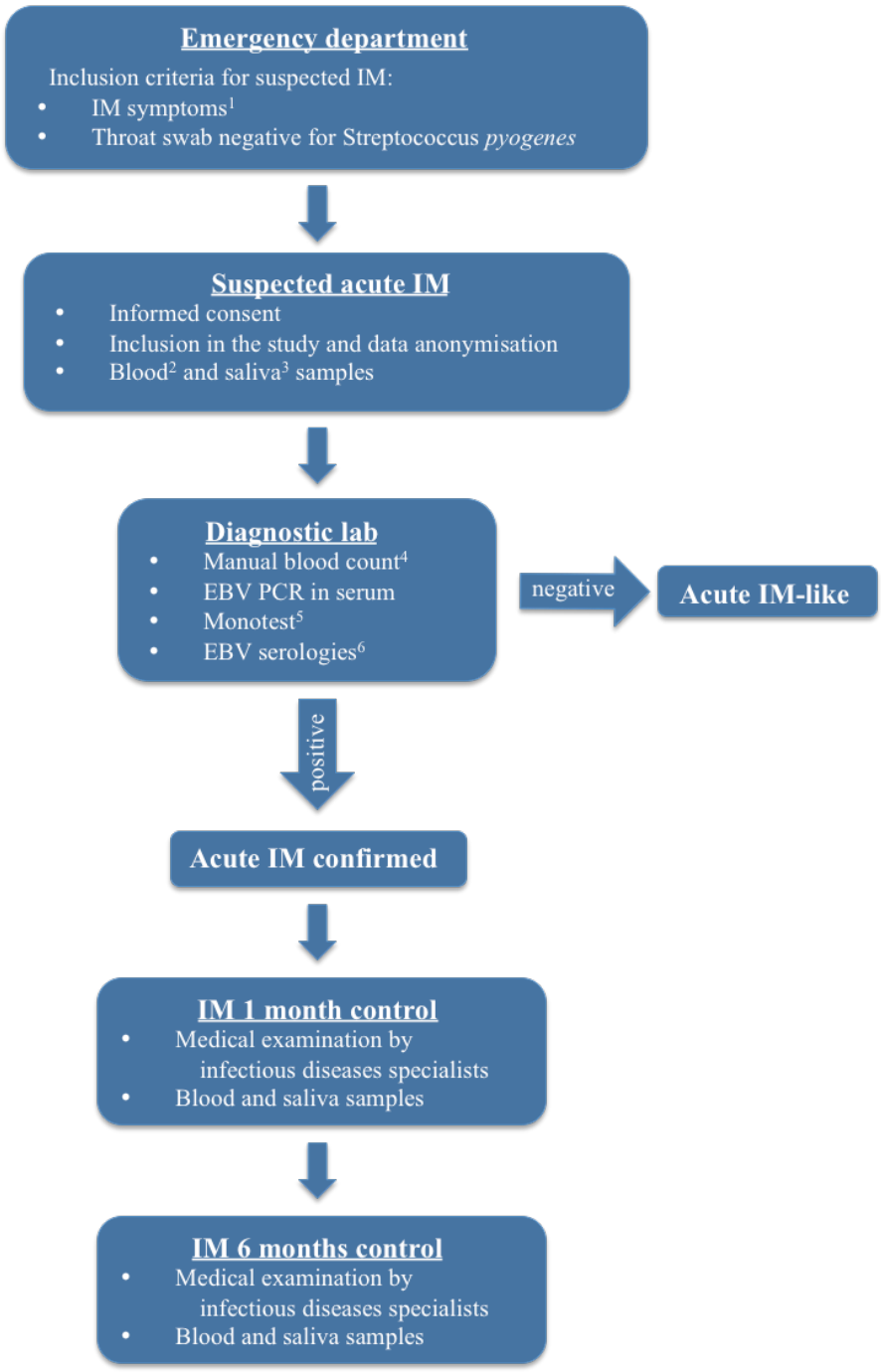
Pediatric patients (age 1-16 years) undergoing elective tonsillectomy for tonsilar hypertrophy or recurrent tonsillitis at the University Children's Hospital of Zurich were invited to participate in the study. Patients and parents were informed about the clinical study protocol orally by the ORL specialists at the preoperative consultation and in writing using a patient information sheet. Informed consent were obtained either from the patient himself depending on his age and from his parents. The tonsils and 2-5 ml of peripheral blood were obtained from each participant during the tonsillectomy. Blood samples were used as controls for flow cytometry analysis.

Healthy adults aged 20 to 30 years working at the University Children's Hospital of Zurich were invited to participate in the study. They were informed about the clinical study protocol orally by the research staff. Co-workers who gave informed consent donated 10-20 ml of peripheral blood. Blood samples were used as controls for flow cytometry analysis.

Ethics approval

This study was conducted in accordance with the Declaration of Helsinki and was approved on 31th May 2010 by the regional ethics committee in Zurich, Switzerland (Reference number EK: StV 40/05).

Clinical study protocol



Footnotes: [1]IM symptoms included fever, tonsillitis, generalized lymphadenopathies, hepatosplenomegaly and fatigue. [2]Blood samples obtained on the emergency department included complete blood counts, serum sample for EBV PCR and serologies, as well as 5-15 ml heparinized peripheral blood for study analysis. [3]Mouth wash samples were obtained after gargling with 5 ml of phosphate-buffered saline. [4]Manual blood counts were used to assess lymphocytosis as well as the presence of atypical lymphocytes (representing activated T cells). [5]Monotest was used to assess the presence of heterophile IgM antibodies. [6]EBV serology was assessed using IgM anti-VCA, IgG anti-VCA and IgG anti-EBNA1.

Role for early-differentiated natural killer cells in infectious mononucleosis

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Running title: NK cell response during acute EBV infection

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Own contribution: I designed the research and performed most of the experiments.

KEY POINTS

- Early-differentiated NK cells accumulate and proliferate during infectious mononucleosis
- These early-differentiated NK cells preferentially target lytic EBV infected B cells in vitro

ABSTRACT

A growing body of evidence suggests that the human natural killer (NK) cell compartment is phenotypically and functionally heterogeneous and composed of several differentiation stages. Moreover, NK cell subsets have recently been shown to exhibit adaptive immune features during herpesvirus infection in experimental mice and to preferentially expand during viral infections in humans. However, both phenotype and role of NK cells during acute symptomatic Epstein-Barr virus (EBV) infection, termed infectious mononucleosis (IM), remain unclear. Here, we longitudinally assessed the kinetics, the differentiation and the proliferation of subsets of NK cells in pediatric IM patients. Our results indicate that acute IM is characterized by the preferential proliferation of early-differentiated $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} NK cells. Moreover, this NK cell subset exhibits features of terminal differentiation and persists at higher frequency over at least the first 6 months after acute IM. Finally, we demonstrate that this NK cell subset preferentially degranulates and proliferates upon exposure to EBV-infected B cells expressing lytic antigens. Thus, early-differentiated NK cells might play a key role in the immune control of primary infection with this persistent tumor-associated virus.

INTRODUCTION

Natural killer (NK) cells are a subset of innate lymphocytes that exhibit non-redundant antiviral functions in experimental mice (105). In mice infected with the murine cytomegalovirus (MCMV), a subset of NK cells bearing the activating receptor Ly49H expands and persists at increased frequency for more than 2 months following primary infection. Notably, these cells display an enhanced protective response against MCMV in adoptive transfer experiments (108). In humans, the peripheral blood compartment of NK cells is heterogeneous and accounts for 5 to 15 % of lymphocytes. It is composed of diverse differentiation stages, which can be defined by the expression of surface markers, such as the two types of inhibitory receptors NKG2A and killer-cell immunoglobulin-like receptors (KIRs) (99, 100). Human NK cells seem to play an important antiviral role since patients with isolated NK cell deficiencies exhibit an increased susceptibility to herpesviruses (110). Furthermore, patients with acute viral infections due to hantavirus, cytomegalovirus, or chikungunya virus (102-104) accumulate the late-differentiated $CD56^{dim}$ $NKG2C^{+}$ KIR^{+} NK cell subset in peripheral blood. However, none of these previous studies demonstrated a protective role for specifically accumulated human NK cell subsets against virus-infected cells *in vitro* or *in vivo* (99, 100).

A ubiquitous persistent human virus, which has not been investigated in detail in this respect, is the primarily B-cell-tropic Epstein-Barr virus (EBV). EBV is a γ-herpesvirus, which latently infects the vast majority of the adult human population worldwide, and is associated with B cell and epithelial cell malignancies (130). EBV displays two modes of infection. One mode expresses latency genes (latent EBV) leading to B cell transformation *in vitro* and subsequent generation of lymphoblastoid cell lines (LCLs). The other mode expresses lytic genes (lytic EBV) leading to production of infectious viral particles and lysis of the host cell (131). Most primary EBV infections occur before the age of 5 years and are

usually asymptomatic. Nevertheless, primary EBV infection occurring beyond this age may manifest as infectious mononucleosis (IM) that affects around 10% of the population in Europe and the US (17, 132). The usually self-limiting IM is characterized by a vigorous $CD8^+$ T cell response that mainly targets EBV lytic epitopes (35) and is associated with an increased risk of developing EBV-positive classical Hodgkin lymphoma (46).

The contribution of particular NK cell subsets to the immune control of EBV, especially during primary infection, remains elusive. Here, we examined how blood NK cell subsets accumulate and respond during IM, and to which extent they can recognize latently and lytically EBV-infected B cells.

MATERIAL AND METHODS

Study design.

Twenty-two pediatric patients diagnosed with acute IM at the University Children's Hospital of Zurich were prospectively enrolled between October 2010 and April 2013. The date of symptoms onset was used as reference for the longitudinal study. Twelve pediatric patients with IM symptoms, but lacking the serological pattern compatible with acute EBV infection, were also enrolled (IM-like) and donated peripheral blood at diagnosis. All serum samples from IM-like patients were negative for HCMV DNA. Healthy children and healthy adults aged 20 to 30 years were used as healthy controls according to their EBV serology. Further details are outlined in supplemental data.

All participants gave informed consent, and the institutional ethics committee approved all protocols used.

Monoclonal antibodies and flow cytometry.

Samples were acquired on a FACSCanto II and a LSR Fortessa (BD Biosciences). Details of handling of PBMCs, flow cytometry analysis and antibodies used are described in supplemental data.

Cell lines.

Preparation of viral stocks, cell lines used and induction and isolation of lytic AKBM cells as well as the degranulation assay are described in supplemental methods.

Viral loads quantification.

EBV DNA levels were determined by real-time PCR. The details of viral load measurements are outlined in the supplemental methods.

Statistical analysis.

Data were analyzed using Prism software (GraphPad Software, Inc.). P-values of < 0.05 were considered significant and calculated with the nonparametric Mann-Whitney test or the Wilcoxon matched-pairs signed ranks tests. Spearman's rank correlation was used to examine associations between two quantitative values.

RESULTS

Pediatric acute IM patients exhibit accumulation of activated $CD8^+$ T cells and $CD56^{dim}$ NK cells.

In young adults, IM is characterized by vigorous T cell responses mediated mainly by EBV-specific $CD8^+$ T cells (35). Nevertheless, neither T cell nor NK cell responses in pediatric IM have been characterized. Since we examined pediatric IM patients only, we first assessed the

dynamics of the T cell and NK cell subset responses in pediatric patients over the first 6 months of IM. Uninfected healthy individuals and pediatric patients with IM-like diseases were used as controls. Acute IM patients exhibited a 2-fold increased median frequency of $CD8^+$ T cells (Figure 1A), a 15-fold increased median frequency of activated $CD8^+$ T cells (Figure 1B) and 60-fold increased median HLA-DR^+ $CD8^+$ T cell counts (data not shown) compared to controls. The numbers of activated T cells normalized within 6 months. These changes paralleled those of the cellular EBV DNA levels over time (Figure 1C). Neither EBV DNA levels nor frequency of $CD8^+$ T cells correlated with age (Supplementary Figure 1). Therefore, pediatric IM patients as young as 2 years of age seem to exhibit the classical immunological features found in young adults with IM.

Moreover, we observed a 1.7-fold increase in the median numbers of NK cells in acute IM compared to controls. These numbers returned to baseline levels after one month (Figure 1D). The blood NK cell compartment is mainly composed of two well-characterized functional subsets, the $CD56^{bright}$ $CD16^-$ and the $CD56^{dim}$ $CD16^+$ subsets (94). The former NK cell subset produces large amounts of cytokines upon monokine stimulation, acquires cytotoxicity only after prolonged activation, and is enriched in secondary lymphoid organs (88). The latter NK cell subset readily kills susceptible targets and can rapidly secrete IFN-g upon engagement of activating receptors (95). Acute IM patients displayed unchanged counts of $CD56^{bright}$ $CD16^-$ NK cells (Figure 1F) and a 1.2-fold increase in the median count of $CD56^{dim}$ $CD16^+$ NK cells (Figure 1H) compared to controls. Interestingly, the intermediate NK cell subset $CD56^{dim}CD16^-$ was increased in frequency during acute IM (Figure 1E and supplementary Figure 2A) and exhibited a 3.7-fold increase in median cell counts compared to controls (Figure 1G). Thus, we found a selective increase of the total $CD56^{dim}$ NK cell subset during acute IM.

Early-differentiated CD56^{dim} NKG2A^{+} KIR^{-} NK cells accumulate during IM and terminally differentiate as well as persist afterwards.

To dissect the CD56^{dim} NK cell subset, we analyzed the expression patterns of the inhibitory receptors NKG2A and KIRs, which might allow assessment of subtle maturation stages spanning from early-differentiated CD56^{dim} NKG2A^{+} KIR^{-} to late-differentiated CD56^{dim} NKG2A^{-} KIR^{+} NK cells (99, 100). Acute IM patients exhibited a 1.8-fold increased median frequency of CD56^{dim} NKG2A^{+} KIR^{-} NK cells (Figures 2A and 2B), but a 1.5-fold reduced median frequency of CD56^{dim} NKG2A^{-} KIR^{+} NK cells compared to EBV-negative and EBV-positive control individuals (Figure 2C). Moreover, acute IM patients displayed 4.8-fold and 3.5-fold increased median absolute numbers of CD56^{dim} NKG2A^{+} KIR^{-} NK cells compared to EBV-negative and EBV-positive control individuals, respectively (Supplementary Figure 2B), but unchanged cell counts of the late-differentiated CD56^{dim} NKG2A^{-} KIR^{+} NK cells. We observed no major changes in the frequencies and cell counts of the CD56^{dim} NKG2A^{-} KIR^{-} and CD56^{dim} NKG2A^{+} KIR^{+} NK cell subsets (Supplementary Figures 2B and 2C). Furthermore, IM-like patients, i.e. patients with IM symptoms but no acute EBV infection, did not exhibit NK cell subset accumulation similar to that of IM patients. We did not find any differences in the frequency of this NK cell subset between EBV-seronegative and EBV-seropositive control individuals. Surprisingly, the frequency of CD56^{dim} NKG2A^{+} KIR^{-} NK cells remained significantly elevated in longitudinally followed IM patients up to 6 months after acute IM, but returned to baseline after 2 years (Figure 2B).

Recently, CD57 has been proposed as a marker of terminal differentiation of NK cells (100, 101) and has been shown to be up-regulated on CD56^{dim} NKG2C^{+} NK cells during acute infection with CMV, hantavirus or chikungunya virus (102-104). Therefore, we hypothesized that if the accumulated CD56^{dim} NKG2A^{+} KIR^{-} subset found in acute IM patients is preferentially involved in the immune response against EBV, it should acquire CD57 during

the acute phase of IM to complete its terminal differentiation. Indeed, we observed a 2.5-fold increase in the median frequency of $CD57^+$ within the $CD56^{dim}$ $NKG2A^+$ KIR^- subset from the acute IM phase to one month later, but no changes in the $CD56^{dim}$ $NKG2A^-$ KIR^+ subset (Figures 2D and 2E, respectively). Thus, acute symptomatic EBV infection elicits the specific accumulation of the $CD56^{dim}$ $NKG2A^+$ KIR^- NK cell subset, its terminal differentiation as well as its persistence at higher frequency over the first 6 months after acute IM.

IM patients exhibit a stable KIR repertoire and unchanged frequencies of $CD56^{dim}$ $NKG2C^+$ NK cells.

The KIR repertoire is composed of several activating and inhibitory receptors specific for distinct groups of HLA class I alleles (76), is highly variable among individuals and is stable over time in healthy adults (133). Particular KIR receptors might be involved in the immune control of viruses such as the human immunodeficiency virus (HIV) (134-137), the hepatitis C virus (HVC) (138, 139) or CMV (133, 140). Therefore, to exclude that IM does not lead to an accumulation of NK cells bearing specific KIRs, we performed a comprehensive phenotypic KIR analysis (141) in a group of CMV-seronegative healthy controls and IM patients. The KIR repertoire of IM patients remained stable over the first month of IM (Figure 3A) and the frequencies of single KIR^+ $CD56^{dim}$ NK cells in IM patients were overall low compared to controls (Figure 3B). Thus, IM is associated with the accumulation of a $CD56^{dim}$ $NKG2A^+$ NK cell subset, which does not carry any increase in activating, nor inhibitory KIR molecule expression. Furthermore, $CD56^{dim}$ NK cells expressing NKG2C, the activating counterpart of NKG2A (78), accumulate upon CMV infection (102, 113, 142), as well as upon other viral infections in CMV-positive individuals (103, 104, 143-145). Thus, we assessed if a similar accumulation takes place upon EBV infection and therefore investigated CMV-seronegative IM patients and controls to avoid bias associated with CMV carriage

(146). We observed no changes in the frequency of NKG2C^{+} NK cells within the CD56^{dim} NKG2A^{-} NK cell subset (Figure 3C and 3D) nor within the expanding CD56^{dim} NKG2A^{+} KIR^{-} NK cell subset (data not shown). Thus, CD56^{dim} NKG2A^{+} KIR^{-} NKG2C^{-} NK cells accumulate during IM.

The preferential proliferation of CD56^{dim} NKG2A^{+} KIR^{-} NK cells positively correlates with cellular EBV loads during acute IM.

We next investigated whether the increase in the absolute numbers of CD56^{dim} NKG2A^{+} KIR^{-} NK cells might be caused by active proliferation of this NK cell subset. We assessed the expression of the proliferation marker Ki-67 in the CD56^{bright}, the CD56^{dim} NKG2A^{+} KIR^{-} and the CD56^{dim} NKG2A^{-} KIR^{+} NK cell subsets in acute IM and one month later. We found a 2-fold and a 3-fold increase in the median frequency of Ki-67^{+} cells within the CD56^{dim} NKG2A^{+} KIR^{-} NK cells in acute IM compared to controls and IM 1 month, respectively (Figures 4A and 4B). However, we did not observe any increased proliferation in the more differentiated NKG2A^{-} KIR^{+} NK subset and there was no difference when comparing EBV-negative with EBV-positive controls (Figures 4A and 4B and data not shown). In addition, the precursor CD56^{bright} NK cell subset, which exhibits strong proliferation properties and responds to minute doses of cytokines *in vitro*, did not show an increased frequency of Ki-67^{+} cells in acute IM patients (Figure 4B). Notably, the frequencies of proliferating Ki-67^{+} cells within the CD56^{dim} NKG2A^{+} KIR^{-} positively correlated with EBV DNA levels in PBMCs (Figure 4C), but not in serum (data not shown). No such correlation was observed within the CD56^{dim} NKG2A^{-} KIR^{+} NK cell subset. This suggested that the proliferation of early-differentiated CD56^{dim} NK cells might be directly driven by EBV-infected B cells in IM patients. We next asked whether the proliferation of the CD56^{dim} NKG2A^{+} KIR^{-} NK cell subset differs according to CD57 expression. Surprisingly, proliferation was exclusively

found within the $CD57^-$ fraction (Fig. 4D and 4E). Our finding is in line with previous studies showing a decreased proliferative potential of $CD57^+$ NK cells compared to $CD57^-$ NK cells (100, 101). Thus, $CD56^{dim}$ $NKG2A^+$ KIR^- $CD57^-$ NK cells seem to preferentially proliferate during acute IM but not one month later. Moreover, in the acute phase, proliferation parallels the accumulation of this NK cell subset which displays a 7-fold and a 4.8-fold median increase compared to EBV-negative and EBV-positive controls, respectively (Figure 4F). In addition, CMV status did not seem to influence the NK cell response (Supplementary Figure 3). We did not observe any correlation between the count of the $CD56^{dim}$ $NKG2A^+$ KIR^- $CD57^-$ NK cells, nor the count of total NK cells, and the EBV DNA levels in PBMCs or in serum (data not shown). Thus, early-differentiated NK cells accumulate in IM patients after EBV-driven proliferation.

$CD56^{dim}$ $NKG2A^+$ KIR^- NK cells preferentially target EBV-infected B cells with lytic reactivation.

$CD56^{dim}$ $NKG2A^+$ KIR^- NK cells are functional against HLA-class-I-deficient target cells, including the EBV-positive LCL 721.221 cell line (99), but their reactivity towards HLA class I competent autologous LCLs has not been assessed yet. We observed a low overall frequency of degranulating NK cells upon co-culture with autologous LCLs. Nevertheless, the $CD56^{dim}$ $NKG2A^+$ KIR^- NK subset, which accumulates during IM, displayed a more than 2-fold increase in degranulation compared to the $CD56^{bright}$ and $CD56^{dim}$ $NKG2A^-$ KIR^+ NK cell subset (Figure 5A). In contrast, the EBV-negative allogeneic B-cell line L428, which exhibits an activated phenotype comparable to LCLs, elicits an increased response in the $CD56^{dim}$ $NKG2A^-$ KIR^+ NK subset only (Figure 5B). This low NK cell response against autologous LCLs might be due to their high surface level of HLA class I and HLA-E which might engage the NK cell inhibitory receptors KIR and NKG2A, respectively. Indeed, EBV-infected B cells up-regulated HLA class I (147) and HLA-E (Figure 5C).

On the other hand, induction of the lytic cycle of EBV infection has been shown to sensitize EBV-infected B cells to NK cell killing using the EBV-positive Akata Burkitt lymphoma (BL) reporter cell line AKBM (121), which allows the purification of BL cells with and without EBV lytic reactivation, respectively. Hence, we tested the degranulation of the $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} NK subset against either latent AKBM or lytic AKBM cells from convalescent IM patients and healthy EBV-positive controls and compared it to the $CD56^{bright}$ and the $CD56^{dim}$ $NKG2A^{-}$ KIR^{+} subsets. In order to avoid HLA class I/KIR mismatch bias, we specifically assessed the degranulation in KIR^{+} matched ($KIR2DL2/DL3/3DL1^{+}$) NK cells according to the AKBM genotype (Bw4/C1/C1). We could confirm increased responses of NK cells against lytic compared to latent AKBM cells (Figure 5D to 5F) previously shown to be mediated by a down-regulation of the inhibitory ligands HLA-class I and HLA-E and an up-regulation of the activating ligands CD112 and ULBP-1 (121). Similarly, we also found increased expression of activating ligands on lytic EBV infected LCLs (Supplementary Figure 4D to 4G), however the expression of the respective activating receptors in the $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} NK cell subset was unaltered in IM patients (Supplementary Figure 4A to 4C). Importantly, the $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} NK cell subset exhibited a significantly stronger degranulation against lytic AKBM cells compared to all other subsets (Figure 5F). We found no difference in the degranulation capacity of $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} NK between convalescent IM patients and controls, suggesting that these NK cells are functional and not in an exhausted state in the aftermath of acute IM. Thus, $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} NK cells preferentially recognize lytic EBV replicating B cells.

EBV lytic replication triggers in vitro proliferation of $NKG2A^{+}$ KIR^{-} NK cells.

Finally, we examined the proliferation of $NKG2A^{+}$ KIR^{-} NK cells using staining for Ki-67 (148) in an *in vitro* model of primary EBV infection of cord blood mononuclear cells (CBMC) infected with either wildtype (WT) EBV or lytic replication incompetent BZLF1-

KO (BZ1KO) EBV. Both viruses elicited similar IFN-type I responses 24 hours post-infection (Figure 6A) and exhibited comparable infection capacity, as evaluated by the frequencies of GFP^+ EBV-infected B cells 3 days post-infection (Figure 6B). Since most NK cells up-regulated CD56 surface expression during *in vitro* culture (Figure 6C, first row), we did not distinguish between the $CD56^{bright}$ and $CD56^{dim}$ NK cells for further analysis. We observed an increased proliferation of $NKG2A^+$ KIR^- NK cells 7 days after infection with WT EBV compared to mock (Figure 6C and 6D). Infection with BZ1KO EBV, with abolished expression of all EBV lytic antigens, elicited a twofold reduced proliferation of $NKG2A^+$ KIR^- NK cells, in comparison to WT EBV (median ratio 2.9 vs. 6.3, P=0.04). This indicates that the proliferation of $NKG2A^+$ KIR^- NK cells partially depends on the presence of EBV-infected cells expressing lytic antigens. Notably, $NKG2A^-$ KIR^+ NK cells exhibit similar proliferation upon infection with WT EBV, but this is not affected by the absence of lytic antigens (data not shown). Finally, we assessed a possible age-dependent distribution of this early-differentiated NK cell subset in peripheral blood from healthy individuals that might correlate with the known age-dependent prevalence of primary symptomatic EBV infection. Indeed, $CD56^{dim}$ $NKG2A^+$ KIR^- NK cells both decreased in frequency (Figure 6E) and in absolute numbers (Figure 6F) over the first decade of life, while the counts of $CD56^{dim}$ $NKG2A^-$ KIR^+ NK cell remained unchanged with age (data not shown). Thus, $CD56^{dim}$ $NKG2A^+$ KIR^- NK cells, which decrease in frequency in the first decade of life, preferentially degranulate and proliferate in response to lytic EBV replicating B cells.

DISCUSSION

Here we demonstrate in longitudinally followed pediatric IM patients that an early-differentiated $CD56^{dim}$ $NKG2A^+$ KIR^- NK subset selectively accumulates during primary symptomatic EBV infection and persists at increased frequencies for months. Moreover, our

data indicates that these NK cells specifically recognize B cells undergoing lytic EBV replication. Our findings are unprecedented and suggest that responses of NK cell subsets to viral infections may not be confined to late-differentiated populations (6-8). Moreover, distinct NK cell subsets may be rather pathogen-specific.

Remarkably, although we found increased counts in the cytotoxic $CD56^{dim}$ NK cell subset, but not in the less differentiated $CD56^{bright}$ $CD16^{-}$ NK cell subset, we could not confirm the previously reported expansion of $CD56^{bright}$ $CD16^{+}$ NK cells during acute symptomatic EBV infection (119). We rather observed an unusual increase of the intermediate $CD56^{dim}$ $CD16^{-}$ NK cell subset, which might be explained by down-regulation of CD56 on $CD56^{bright}$ NK cells or by down-regulation of CD16 such as observed in degranulating $CD56^{dim}$ NK cells upon co-culture with K562 (unpublished observations). This $CD56^{dim}$ NK cell subset is distinctly characterized by NKG2A expression and by the absence of KIRs. It strikingly differs from that of other acute viral infections such as CMV, hantavirus, or chikungunya virus infection (102-104), in which the $CD56^{dim}$ KIR^{+} $NKG2C^{+}$ NK cell subset was shown to be expanded. $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} NK cells are considered early-differentiated (99) as suggested by the specific temporal reconstitution of the NK cell subsets in hematopoietic stem cell transplanted patients (149-151) and in mice with human immune system components (100). Based on our results, ongoing differentiation of these early-differentiated NK cells seems to occur during the first weeks of IM; this is further supported by studies in EBV-infected mice with human immune system components (62).

Another striking feature of the IM-associated NK cell response is the persistence of elevated frequencies of the $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} NK cells for up to 6 months long after $CD8^{+}$ T cell numbers have normalized. Nevertheless, we observed no difference in the peripheral blood frequencies of these NK cells between EBV-seropositive and EBV-seronegative control individuals, contrasting the situation after CMV infection where late

differentiated NK cells persist at increased levels (113). This might be explained by compartmentalized NK cell accumulation during asymptomatic EBV infection, as has been proposed for EBV-specific T cell responses in the tonsils (48, 49) and suggests that the acute symptomatic EBV infection systemically imprints the NK cell compartment differently. Accordingly, $CD56^{bright}$ $NKG2A^{+}$ and mostly KIR^{-} NK cells were found enriched in tonsils of EBV-seropositive compared to EBV-seronegative individuals (16). Indeed, this could result from ongoing lytic EBV replication at these sites in asymptomatic EBV carriers (62). Additionally, we determined that the increased $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} NK cell numbers during acute IM were caused by the selective proliferation of this subset. Such selectivity has not been reported to our knowledge during other acute viral infections. Since the $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} NK subset only actively proliferated during the acute phase of IM and was only increased in absolute numbers at this stage, the persistently increased frequency of this subset might be caused by either a longer survival of these NK cells in peripheral blood or a continuous EBV-driven proliferation in tissues followed by recruitment and accumulation in the peripheral blood. Indeed, the numbers of EBV-infected B cells quickly decrease in the peripheral blood after acute IM (23) and EBV turns off antigen expression in these cells (24). Thus, an EBV-driven proliferation of NK cells during IM convalescence might not be expected in peripheral blood. Nevertheless, IM patients exhibit prolonged oral EBV shedding up to one year after IM (17, 31), indicating that EBV replication takes place in the oropharynx. This, in turn, may result in local EBV-driven proliferation of NK cells after IM that are subsequently recruited in peripheral blood.

Several lines of evidence support the hypothesis that EBV-infected B cells, and not proinflammatory cytokines, directly drive the unique proliferation of early-differentiated NK cells: firstly, a similar expansion is not observed in patients with IM-like symptoms; secondly, the precursor $CD56^{bright}$ NK cell subset, which possesses strong proliferative responses to

cytokines, does not display an enhanced proliferation during acute IM; thirdly, the frequency of proliferating early-differentiated NK cells positively correlates with EBV loads in blood cells. On the other hand, we did not observe any correlation between overall NK cell counts and viral load. These findings are in conflict with previous studies reporting a negative correlation between NK cell frequencies and counts and cellular EBV DNA levels (119) or a positive correlation between NK cell counts and EBV DNA levels in whole blood (17). Hence, acute IM elicits the proliferation of $CD57^-$ $CD56^{dim}$ $NKG2A^+$ KIR^- NK cells, which then differentiate to become CD57 positive. A fraction of this subset might represent antigen-experienced NK cells that specifically responded to EBV-infected cells in a similar fashion as during MCMV infection of experimental mice where these NK cells are suggested to constitute memory NK cells (108).

These early-differentiated NK cells exhibit enhanced degranulation against EBV-infected B cells with lytic reactivation. Reduced HLA-E-mediated inhibitory signals on EBV-infected cells expressing lytic antigens might lead to preferential recognition by $NKG2A^+$ NK cells and could be caused by decreased availability of class I leader signal peptide (121) or via direct modulation of HLA-E by EBV-derived peptides (152). In addition to these diminished inhibitory signals, we and others have observed an increased expression of activating ligands in lytic EBV infection (17) and NKG2D and DNAM1 have been identified as the main activating NK cell receptors for the recognition of B cells with lytic EBV infection in vitro (17). In good agreement, loss of NKG2D function has been recently found to confer susceptibility to uncontrolled EBV infection and neoplasia in XMEN patients in vivo (18). How lytic EBV infection leads to the up-regulation of activating ligands, however, remains unclear. NKG2D ligands have been reported to get up-regulated upon induction of the DNA damage response and cytosolic DNA recognition (63). Although EBV like other herpesviruses packages its DNA into the viral capsid in the nucleus during lytic replication,

some of it might become accessible during capsid transit through the cytosol and trigger this pathway of NKG2D ligand up-regulation. Newly infected B cells transiently express several lytic antigens, mostly from the immediate early and early lytic genes (153), and some of these antigens might contribute to the proliferation of $NKG2A^+$ KIR^- NK cells upon EBV infection of CBMCs.

We suggest that $CD56^{dim}$ $NKG2A^+$ KIR^- NK cells preferentially recognize autologous B cells with lytic EBV infection, and that recognition of lytic EBV replication drives the characteristic NK cell accumulation that we observed in acute IM patients. Since IM only manifests in older children, adolescents and young adults with decreased frequencies of this herein newly described EBV-reactive NK cell subset, we postulate an age-dependent impaired NK cell-mediated immune control of EBV infection as one possible cause of IM.

Authorship Contributions

T.A. designed and performed research; A.L., A.M., V.B. and K.M. performed experiments and analyzed data; C.M., O.C. and D.N. designed research; S.U., G.S., C.G., and C.B. contributed essential information or vital reagents; T.A. and O.C. analyzed data; and T.A., C.M., O.C. and D.N. wrote the manuscript.

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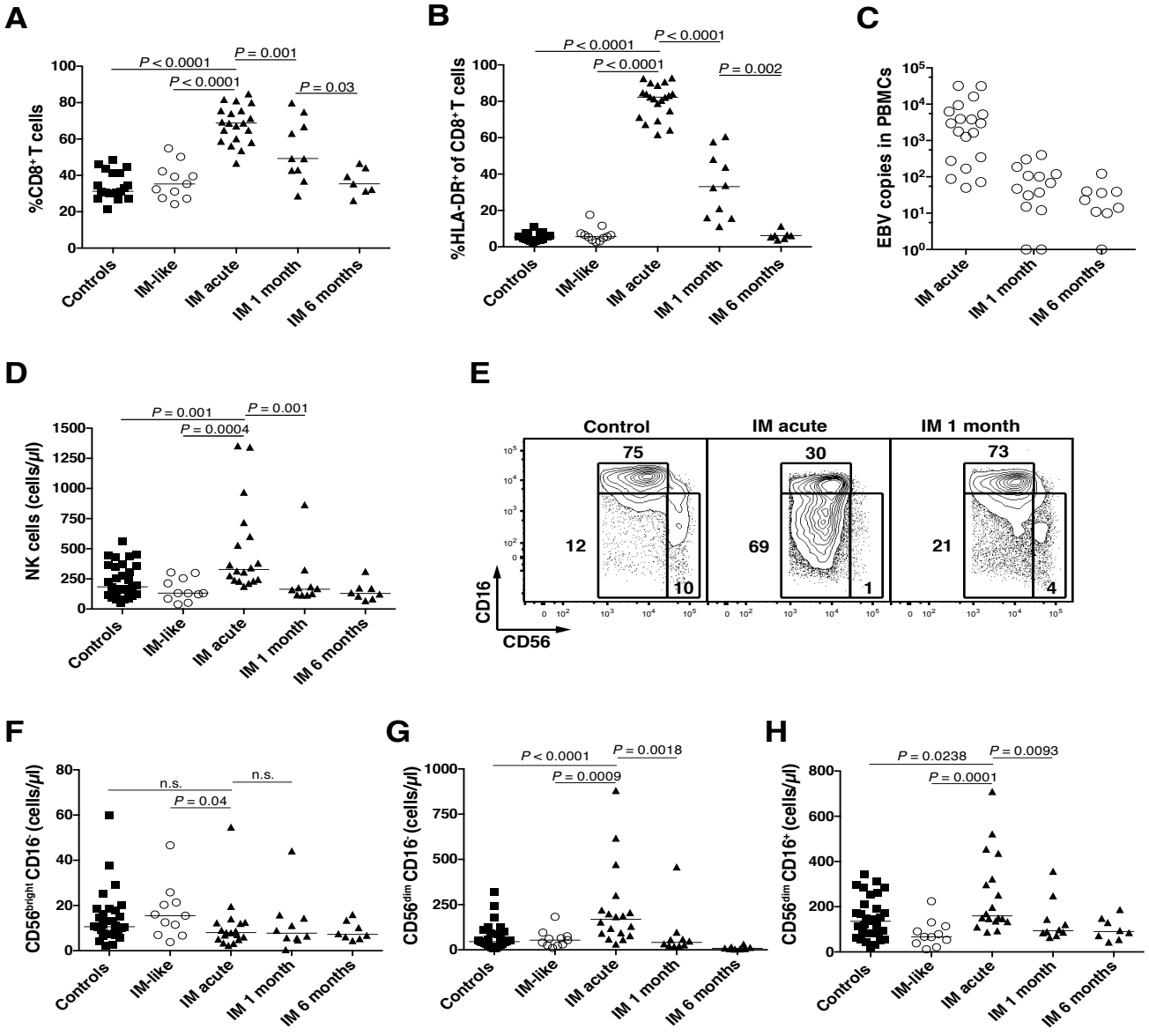


Figure 1. Accumulation of activated HLA-DR$^+$ CD8$^+$ T cells and CD56^{dim} NK cells during acute IM. PBMCs from healthy controls, IM-like patients and IM patients at acute phase (IM acute), at 1 (IM 1 month) and 6 months (IM 6 months) were analyzed by flow cytometry. Frequencies of (**A**) CD8$^+$ T cells within the CD3$^+$ T cell population and (**B**) HLA-DR$^+$ CD8$^+$ T cells within the CD8$^+$ T cell population in healthy controls (n=19), IM-like (n=11) and IM acute (n=20), 1 month (n=10) and 6 months (n=7) patients. (**C**) EBV DNA load in copies per 10^6 PBMCs in IM acute (n=19), 1 month (n=14) and 6 month (n=9) patients. Counts (cells/μl blood) of total NK cells (**D**) and frequencies of CD56^{bright} CD16$^-$, CD56^{dim} CD16$^-$ and CD56^{dim} CD16$^+$ NK cell subsets within the CD3$^-$ CD56$^+$ NK cell population from representative healthy control, IM acute and 1 month patient (**E**). Counts (cells/μl blood) of (**F**) CD56^{bright} CD16$^-$, (**G**) CD56 ^{dim} CD16- and (**H**) CD56^{dim} CD16$^+$ NK cells in healthy controls (n=31), IM-like patients (n=11) and IM acute (n=18), 1 month (n=10) and 6 months (n=8) patients.

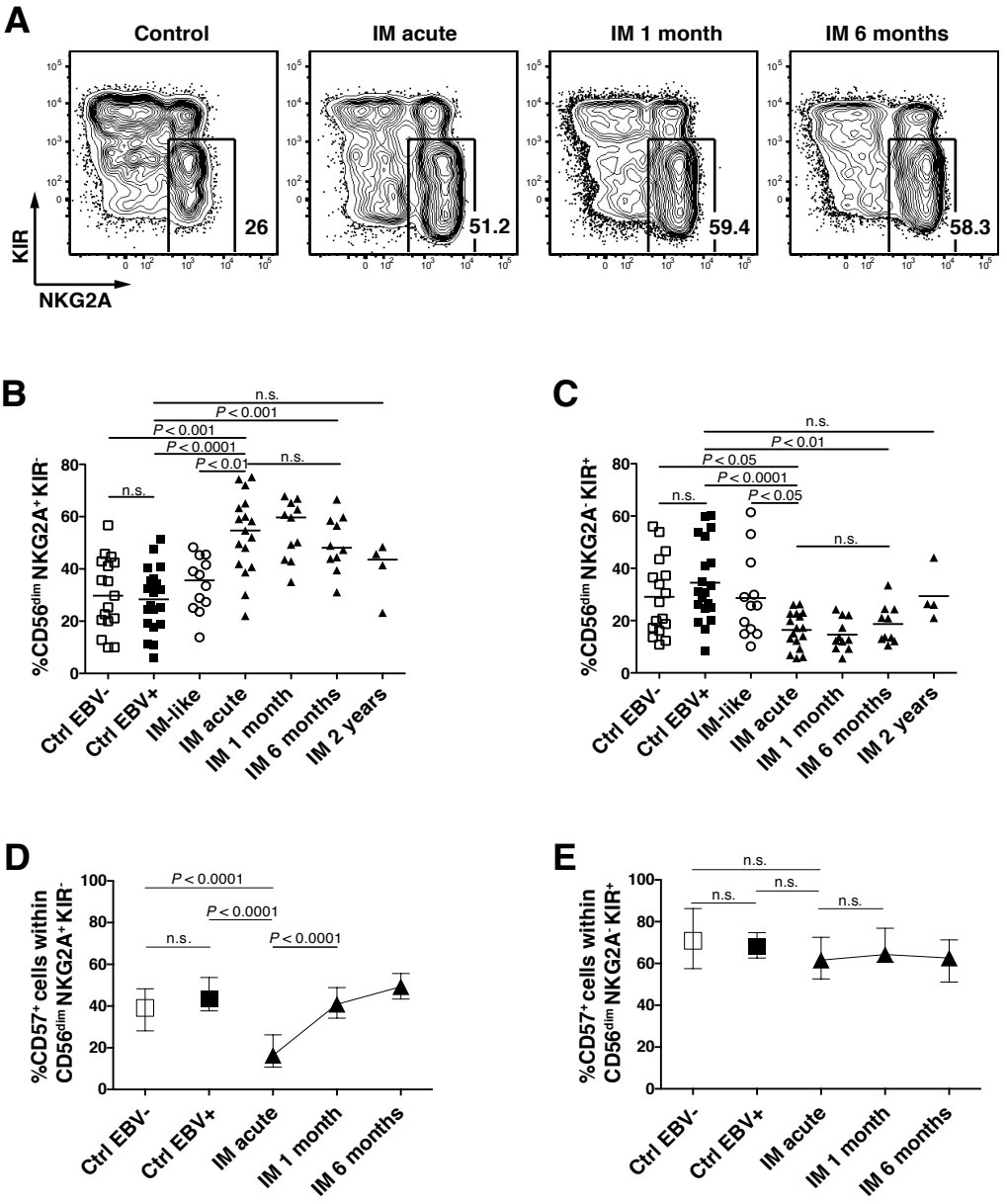


Figure 2. Accumulation and terminal differentiation of the CD56^{dim} NKG2A^{+} KIR^{-} NK cell subset during acute IM. PBMCs from controls and IM patients were analyzed by flow cytometry. (**A**) Frequencies of CD56^{dim} NKG2A^{+} KIR^{-} NK cells within the CD56^{dim} population from representative healthy control, IM acute, 1 month and 6 months patients. Frequencies of (**B**) CD56^{dim} NKG2A^{+} KIR^{-} and (**C**) CD56^{dim} NKG2A^{-} KIR^{+} NK cells in healthy EBV-negative controls (n=17), healthy EBV-positive controls (n=20), IM-like patients (n=12) and IM acute (n=17), 1 month (n=11), 6 months (n=10) and 2 years (n=4) patients. Frequencies of CD57^{+} cells within (**D**) the CD56^{dim} NKG2A^{+} KIR^{-} and (**E**) the CD56^{dim} NKG2A^{-} KIR^{+} NK cells subsets in healthy EBV-negative controls (n=17), healthy EBV-positive controls (n=20) and IM acute (n=17), 1 month (n=11), 6 months (n=10) and 2 years (n=4) patients. Horizontal lines or single symbols indicate median values. Error bars indicate interquartile ranges. Mann-Whitney tests.

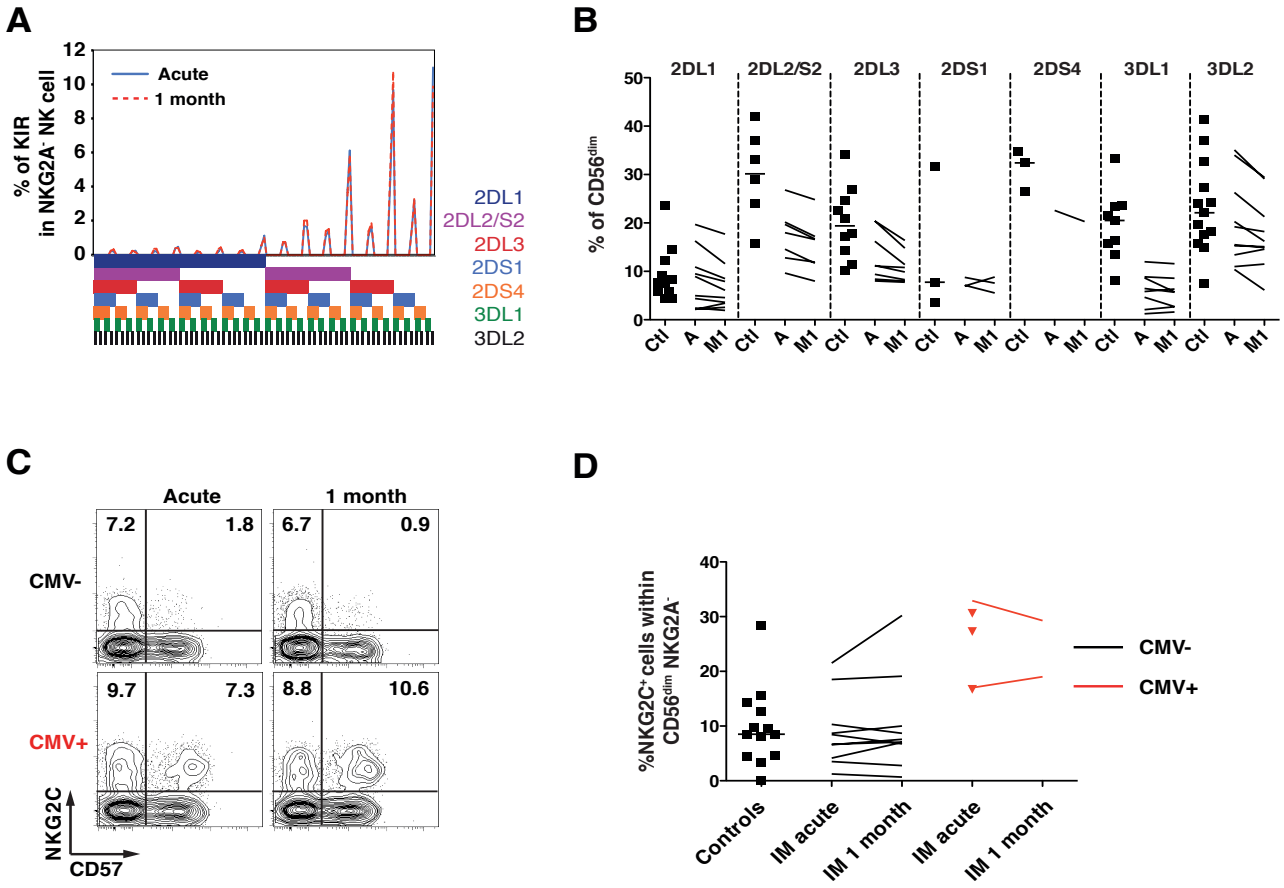


Figure 3. Frequencies of single KIR-positive and NKG2C$^+$ CD56^{dim} NK cells are not altered during acute IM. (**A**) Frequencies of CD56^{dim} NKG2A$^-$ NK cells expressing the 7 analyzed KIRs from one representative CMV-seronegative IM patient at acute phase and at 1 month. The presence of one KIR in a combination is represented by a color code below the graph. (**B**) Frequencies of single KIR-positive CD56^{dim} NK cells in healthy controls (Ctl, n=11) and IM patients (n=10) at acute phase (A) and at 1 month (M1). (**C**) Frequencies of NKG2C$^+$ CD57$^+$ NK cells within the CD56^{dim} NKG2A$^-$ population from one CMV-seronegative and one CMV-seropositive IM patient at acute phase and at 1 month. (**D**) Frequencies of NKG2C$^+$ NK cells within the CD56^{dim} NKG2A$^-$ NK cell population in CMV-seronegative healthy controls (n=13), CMV-seronegative (n=10) and CMV-seropositive (n=5, red) IM patients at acute phase and at 1 month (n=2 for CMV-seropositive, red lines).

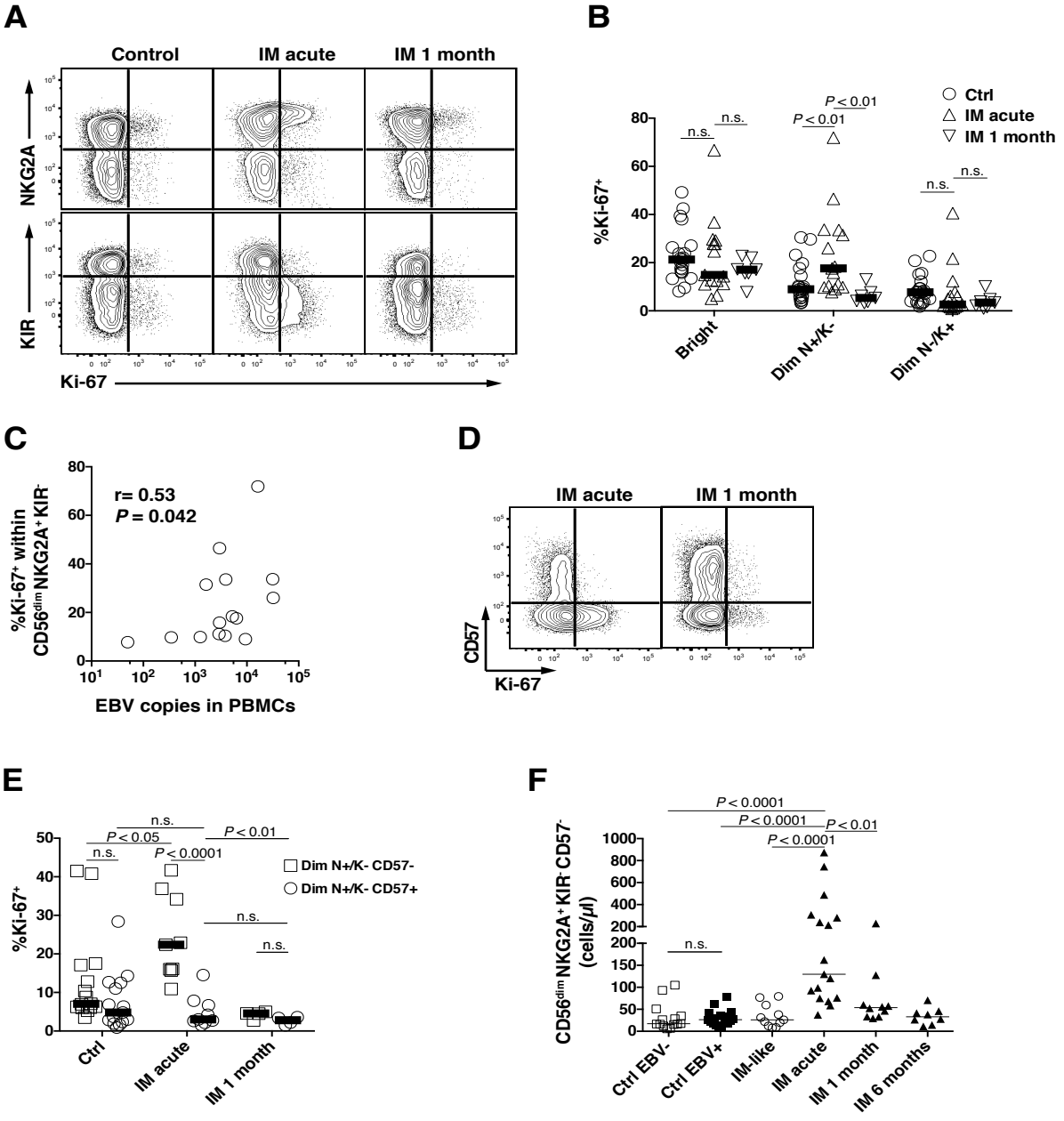


Figure 4. Increased count of CD56^{dim} NKG2A^{+} KIR^{-} CD57^{-} NK cells during acute IM is caused by preferential proliferation. (**A**) Representative examples of co-staining for NKG2A and Ki-67 and co-staining for KIR and Ki-67 on CD56^{dim} NK cells in healthy control and IM acute and 1 month patients. (**B**) Frequencies of Ki-67^{+} cells within the CD56^{bright}, CD56^{dim} NKG2A^{+} KIR^{-} and CD56^{dim} NKG2A^{-} KIR^{+} NK cell subsets in healthy controls (n=21), in IM acute (n=15) and 1 month patients (n=7). (**C**) Correlation of EBV DNA loads (copies per 10^{6} PBMCs) and frequencies of Ki-67^{+} cells within the CD56^{dim} NKG2A^{+} KIR^{-} NK cell subset from acute IM patients. Spearman r = 0.53, P (two-tailed) = 0.042. (**D**) Representative example of co-staining for CD57 and Ki-67 on CD56^{dim} NKG2A^{+} KIR^{-} NK cells in an IM acute and 1 month patient. (**E**) Frequencies of Ki-67^{+} cells within the CD56^{dim} NKG2A^{+} KIR^{-} NK cells according to CD57 expression in healthy controls (n=15), IM patients acute (n=9) and 1 month (n=4) patients. (**F**) Count of CD56^{dim} NKG2A^{+} KIR^{-} CD57^{-} in healthy EBV-negative controls (n=14), healthy EBV-positive controls (n=17), IM-like (n=11), IM acute (n=17), 1 month (n=10) and 6 months (n=8) patients. Horizontal lines indicate median values of a given symbol. Mann-Whitney tests.

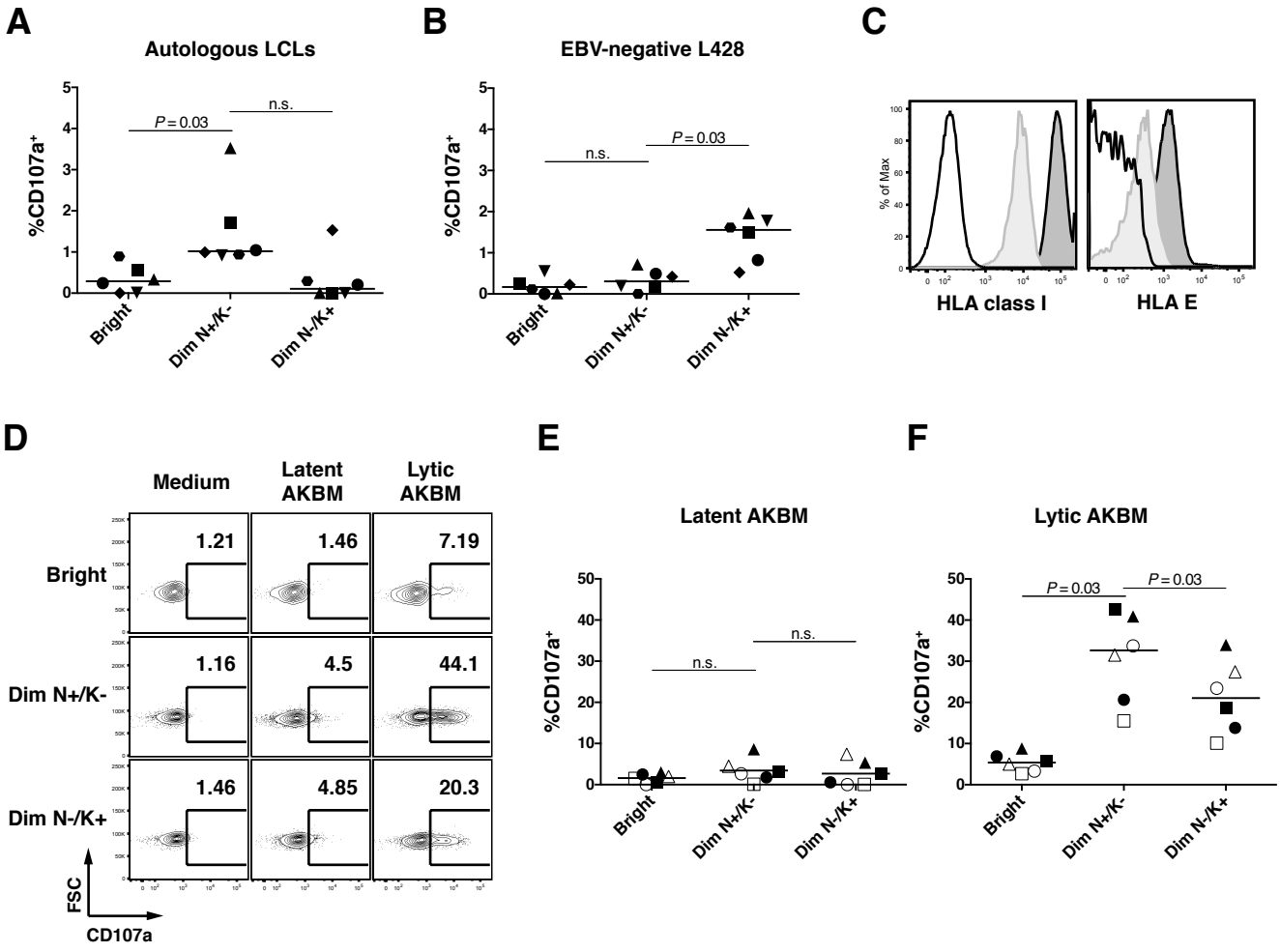


Figure 5. Increased cytotoxic degranulation of the CD56^{dim} NKG2A^{+} KIR^{-} NK subset against EBV-infected B cells with lytic replication. (**A**) PBMCs from 6 healthy EBV-positive donors were co-cultured with autologous LCLs and (**B**) EBV-negative L428 at an effector to target ratio of 10:1 for 6 hours. Frequencies of degranulating (CD107a^{+}) cells within the CD56^{bright} (Bright), the CD56^{dim} NKG2A^{+} KIR^{-} (Dim N+/K-) and the CD56^{dim} NKG2A^{-} KIR^{+} (Dim N-/K+) NK cell subsets were assessed by flow cytometry at the end of the co-culture. (**C**) HLA class I and HLA-E expression on CD19^{+} B cells from PBMCs (light gray histogram) and from autologous LCLs (dark gray histogram). Isotype controls are depicted as white histogram. (**D**) Representative example of frequencies of CD107a^{+} NK cells within the 3 NK cell subsets after co-culture with latent AKBM or lytic AKBM. Frequencies of degranulating (CD107a^{+}) NK cells within the CD56^{bright} (Bright), the CD56^{dim} NKG2A^{+} KIR^{-} (Dim N+/K-) and the CD56^{dim} NKG2A^{-} matched KIR^{+} (Dim N-/K+) NK cell subsets in PBMCs from 3 convalescent IM patients (open symbols) and 3 healthy EBV-positive donors (filled symbols) co-cultured with (**E**) latent AKBM or (**F**) lytic AKBM (n=6). Horizontal lines indicate median values of a given subset, Wilcoxon matched-pairs signed ranks tests.

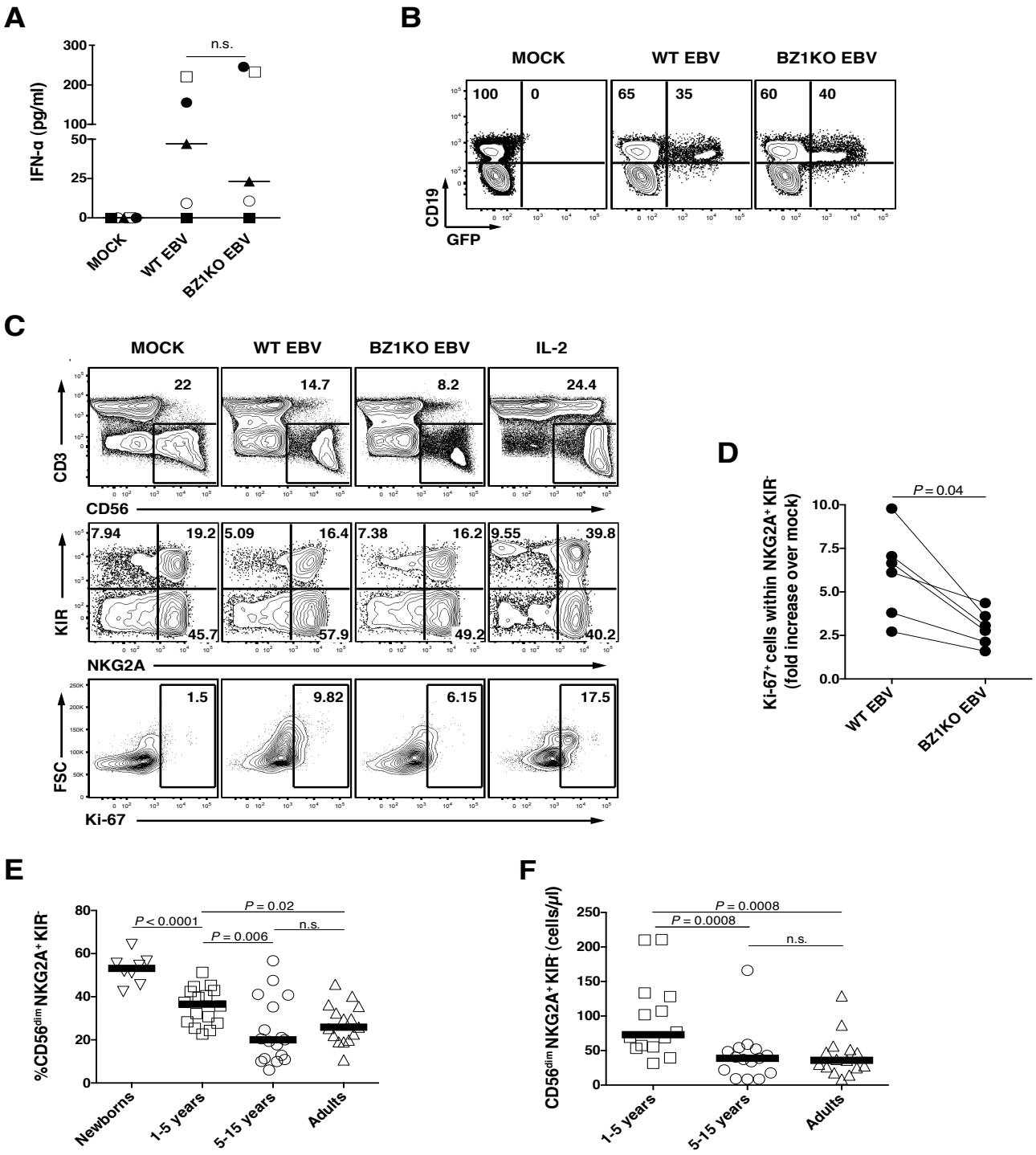


Figure 6. EBV-driven *in vitro* proliferation of NKG2A⁺ KIR⁻ NK cells partially depends on expression of lytic antigens. Proliferation of NK cell subsets was assessed 7 days after inoculation of CBMCs with either wildtype (WT) EBV, BZLF1-KO (BZ1KO) EBV or PBS (MOCK). (**A**) Concentrations of IFN-α in supernatant 24 hours post-inoculation upon MOCK, WT EBV and BZ1KO EBV infection (pg/ml; n=5). (**B**) Representative example of CD19 and GFP co-staining within live lymphocytes 72 hours post-infection. Numbers indicate frequencies of GFP-negative or GFP-positive cells within the CD19⁺ B cell population. (**C**) Frequencies of Ki-67⁺ NKG2A⁺ KIR⁻ NK cells 7 days after inoculation of CBMCs with mock, WT EBV or BZ1KO EBV or after stimulation with IL-2. The depicted gates were assessed within live lymphocytes (first row), CD3⁻ CD56⁺ NK cells (second row) and NKG2A⁺ KIR⁻ NK cells (third row). (**D**) Ratio of NKG2A⁺ KIR⁻ Ki-67⁺ NK cell counts from WT EBV- or BZ1KO EBV- over mock-infected CBMCs. (**E**) Frequencies of $CD56^{dim}$ NKG2A⁺ KIR⁻ NK cells in newborns (n=8), children aged 1-5 years (n=16), children aged 5-15 years (n=17) and adults aged 20-30 years (n=15). (**F**) Counts of $CD56^{dim}$ NKG2A⁺ KIR⁻ NK cells in children aged 1-5 years (n=14), children aged 5-15 years (n=15) and adults aged 20-30 years (n=15). Horizontal lines indicate median values of a given age group, Mann-Whitney tests.

SUPPLEMENTARY DATA

Material and methods

Study design and human samples.

Twenty-two pediatric patients diagnosed with acute IM at the University Children's Hospital of Zurich were prospectively enrolled between October 2010 and April 2013. The date of symptoms onset was used as reference for the longitudinal study. Heparinized peripheral blood samples were obtained from IM patients at diagnosis (IM acute), as well as for some study patients at one (IM 1 month) six months (IM 6 months) and 2 years (IM 2 years) after the first medical assessment. IM patients with duration of symptoms of < 4 weeks were included in the IM acute group. Two IM patients with symptoms lasting > 4 weeks were included in the IM 1 month group. Diagnosis of acute IM was established on the basis of the presence of IM symptoms such as fever, tonsillar enlargement, hepatosplenomegaly and generalized lymph node enlargement, plus detectable immunoglobulin M (IgM) to viral capsid antigen (VCA) and absent immunoglobulin G (IgG) to EBV nuclear antigen 1 (EBNA 1) in serum (Immuno-DOT; GenBio). Twelve pediatric patients with IM symptoms, but lacking the serological pattern compatible with acute EBV infection, were also enrolled (IM-like) and donated peripheral blood at diagnosis. All serum samples from IM-like patients were negative for HCMV DNA by qPCR analysis as well as negative for IgM to HCMV (Immuno-DOT; GenBio).

Heparinized peripheral blood samples from healthy children undergoing elective tonsillectomy and healthy adults aged 20 to 30 years were obtained. These individuals were used as healthy controls according to their EBV serology. EBV carriers were defined as EBV-seropositive individuals (presence of IgG to VCA in serum). Acute EBV and CMV infections were excluded in healthy controls by the absence of detectable IgM to VCA and IgM to HCMV in serum samples (Immuno-DOT; GenBio).

All participants gave informed consent, and the institutional ethics committee approved all protocols used.

Sample collection and handling.

Peripheral blood samples were centrifuged and serum was collected and frozen at –80°C for later use. PBMCs were isolated by density gradient centrifugation (Ficoll-Paque PLUS, GE

Healthcare) per the manufacturer's instructions and frozen at –160°C. Peripheral blood volumes and yielded PBMCs counts were recorded and used for calculation of NK cell subsets counts. Cell counts of cord blood mononuclear cell (CBMC) samples were not available.

Monoclonal antibodies and flow cytometry.

The following monoclonal antibodies (mAbs) were used (clone names are given within parentheses): anti-CD56 (NCAM 16.2) PE-Cy7, anti-CD107a (H4A3) FITC, anti-Ki-67 (B56) FITC, anti-CD57 (NK1) FITC, anti-CD4 (RPA-T4) FITC, anti-CD8 (HIT8a) PE (BD Pharmingen); anti-CD159a or NKG2A (Z199) PE, anti-CD158a, h or KIR2DL1/2DS1 (EB6B) APC and PC5.5, anti-CD158b1/b2, j or KIR2DL2/2DL3/2DS2 (GL183) APC (Beckman Coulter); anti-KIR3DL1 (DX9) APC (R&D systems); anti-CD3 (UCHT1) pacific blue (Invitrogen); anti-CD3 (HIT3a) APC-Cy7, anti-CD57 (HCD57) pacific blue, anti-CD16 (3G8) APC-Cy7, anti-HLA-DR (L243) APC-Cy7, anti-CD19 (HIB19) pacific blue, anti-CD107a (H4A3) pacific blue (BioLegend); anti-HLA-ABC (clone W6/32) FITC, anti-HLA-E (clone 3D12HLA-E) PE (eBioscience), anti-NKG2D (1D11) APC, anti-DNAM-1 (clone11A8) and anti-2B4 (C1.7) FITC, anti-EBV ZEBRA (BZ1) unlabeled (Santa Cruz Biologicals), anti-CD155/PVR (TX21) PE (Acris Antibodies), anti-PVRL/CD112 (TX31) (LifeSpan Biosciences), anti-CD48 (248) PE (SinoBiologicals). Additionally, the following polyclonal antibody was used: rabbit anti-human MICA PE (LifeSpan Biosciences). Pan-KIR staining was performed with a combination of KIR2DL1/2DS1, KIR2DL2/2DL3/2DS2 and KIR3DL1 mAbs (all in APC).

For phenotypic analyses, frozen PBMCs were thawed, washed and stained with mAbs at 4°C for 30 minutes. LIVE/DEAD Fixable Aqua (Invitrogen) was used for dead cell exclusion in every flow cytometry analysis. Intracellular staining was performed after fixation and permeabilization with the BD Cytofix/Cytoperm Kit (BD Biosciences) according to the manufacturer's protocol. Samples were acquired on a FACSCanto II or a LSR Fortessa (both BD Biosciences) and all flow cytometry analyses were performed with FlowJo Version 9 software (Tree star, Inc).

The comprehensive single KIR analysis was performed using the following mAbs:

KIR2DL3 (180701) FITC, NKG2A (Z199) APC, KIR3DL1 (DX9) Brilliant violet 421, CD57 (TB01) functional grade purified, CD14 (clone M5E2) Horizon-V500, CD19 (HIB19) Horizon-V500, KIR2DS4 (179315) Qdot.585, KIR3DL2 (DX31) biotin, KIR2DL1 (143211) Alexa fluor 700, NKG2C (134522) PE, CD56 (N901) ECD, CD3 (UCHT1) PE-Cy5,

KIR2DL2/S2/L3 (GL183) PE-Cy5.5, KIR2DL1/S1 (EB6) PE-Cy7. Biotin-conjugated antibodies were visualized using streptavidin-Qdot 605. CD57 was visualized using an anti-mouse IgM (II/41) eFluor 650. KIR2DS4 was conjugated in house with Qdot 585 and Qdot 705 conjugation kits (Invitrogen), respectively. Samples were acquired on a LSR Fortessa (BD Biosciences).

Preparation of virus stock and generation of autologous lymphoblastoid cell lines (LCLs).

The EBV-infected marmoset B95-8 cells were seeded at a density of 106 cells/ml and were stimulated to release virus by being cultured for 4 days in RPMI 1640 medium (Sigma-Aldrich) with 10% heat-inactivated fetal bovine serum (Life Technologies), 1% L-glutamine and 1% penicillin-streptomycin (referred to hereafter as R10) containing 50 ng of 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma-Aldrich)/ml. Cell suspensions were centrifuged at 1000 x g for 10 min. Supernatant was passed through a 0.45-µm-pore-size cellulose acetate filter (Millipore, Zug, Switzerland) and stored at −80°C. The cell-free supernatants contained approximately 1 x 108 EBV DNA copies/ml as evaluated by real-time PCR.

Freshly isolated PBMCs from EBV-positive healthy adult donors and IM subjects were infected with the B95-8 EBV at a MOI of 0.2. Cyclosporin A (400 ng/ml; Sigma-Aldrich) was immediately added to inhibit the killing of EBV-infected cells by EBV-specific T cells. The cells were seeded on a 96-wells plate and kept in culture for at least 6 weeks before use as target cells in degranulation assays.

Recombinant GFP-Epstein-Barr virus (EBV) B95.8 wildtype and BZLF-1 KO were produced in 293 cells (kindly provided by Prof. Henri-Jacques Delecluse) as described elsewhere (154). Titration of viral concentrates was done on Raji cells in serial dilutions and calculated as Raji infecting units (RIU) using flow cytometric analysis of GFP-positive Raji cells 2 days after infection of cells.

Cell lines.

The B95-8 marmoset cell line, the EBV negative L428 B cell line, established LCLs, K562 and the Raji cell lines were maintained in R10. LCLs for analysis of NK cell activating ligands were kept for one week without medium change before being analyzed. AKBM cells (genotype HLA A 24/31, B 35/51; C 3/14) were maintained under selection in R10 with 200µg/ml Hygromycin B (HygroGold, InvivoGen). 293 cells were maintained in D10 with 100µg/ml Hygromycin B.

Isolation of AKBM cells in lytic cycle.

The induction of the lytic cycle in AKBM cells and their purification were done as described before (17). Briefly, AKBM cells were stimulated with 100µg/ml polyclonal rabbit anti-human F(ab) IgG (DAKO) for 2 hours at 37°C, washed and further cultured at 37°C. After 24 hours, AKBM cells in lytic cycle expressing the rat CD2 on the surface were purified by MACS sorting (Miltenyi Biotec) using the mouse anti-rat CD2 antibody (clone OX34; Abcam) and the anti-mouse IgG2a+b Microbeads (Miltenyi Biotec). Both the purified lytic and latent AKBM cells were used directly in degranulation assays.

Degranulation assay.

To evaluate the cytotoxic degranulation of NK cells, frozen PBMCs from IM patients and EBV-positive healthy donors were thawed and rested overnight and 10^6 PBMCs were co-cultured the next day with target cells at an effector to target ratio of 10:1 for 6 hours in the presence of anti-CD107a antibody. To detect spontaneous degranulation, a control without target cells was included. After 1 hour, monensin (1µg/ml BD Golgi Stop; BD Pharmingen) was added to all samples. At the end of the incubation, cells were stained with mAbs and analyzed by flow cytometry. The spontaneous degranulation (background) from NK cell subsets was subtracted in each degranulation analysis.

Infection of cord blood mononuclear cells with recombinant EBV and NK cell proliferation assay.

Cord blood mononuclear cells (CBMCs) depleted from CD34+ cells (kindly provided by Prof. Roberto Speck) were thawed and rested overnight in R10. The next day, CBMCs were counted, resuspended in R10 at a concentration of 2x 10^6 cells/ml and inoculated for 1 hour at 37°C with either mock, WT EBV or BZ1KO EBV at an MOI of 0.5 (106 RIU/ml). After inoculation, CBMCs were washed, resuspended in R10 at a concentration of 2 x 10^6 cells/ml and 0.5 x 10^6 cells were plated in duplicate wells of a U-bottom 96-well plate and incubated at 37°C for 7 days. IL-2-stimulated CBMCs (100U/ml; Peprotech) were used as positive control for NK cell proliferation. After 24 hours, 100µl of supernatant were removed for further quantification of IFN-α by ELISA (Mabtech) and replaced with R10. After 7 days, CBMCs were counted, washed, stained with mAbs and analyzed by flow cytometry to assess proliferation of NK cell subsets.

Viral loads quantification.

DNA was extracted from frozen PBMCs, and after addition of herring sperm DNA (Life Technologies) from sera, using the QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. EBV DNA levels were determined by the TaqMan (Applied Biosystems) real-time PCR technique targeting a 101-base pair sequence conserved in the EBV BamHI W region (155). PCR primers (Microsynth) were designed with the following sequences: 5'-GCCAGACAGCAGCCAA-TTGT-3' and 5'-GACTCCTGGCGCTCTGATG-3'. Within this region, the hybridization probe was designed with the sequence 5'-FAM-ACTGCCCCTGGTATAAAGTGGTCC-TAMRA-3' (Applied Biosystems). Amplification of DNA sequences was performed using TaqMan® Gene Expression Master Mix (Applied Biosystems) and was analyzed using an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). Serial dilutions of a pUC18 plasmid containing the targeted sequence were included in every PCR run as an internal control and for calibration. To quantify the EBV DNA copy numbers in 106 PBMCs, the BamHI Ct values were normalized to the Ct value of the single-copy human hydroxymethylbilane synthase (HMBS) gene. Amplification was done with the primers: 5'-GCTCGCATACAGACGGACAG-3' and 5'-AGGCCAGGGTACGAGGCTT-3' and the probe 5'FAM-TGGTGGCAACATTG-MGB-3' (Applied Biosystems).

SUPPLEMENTARY FIGURES

A

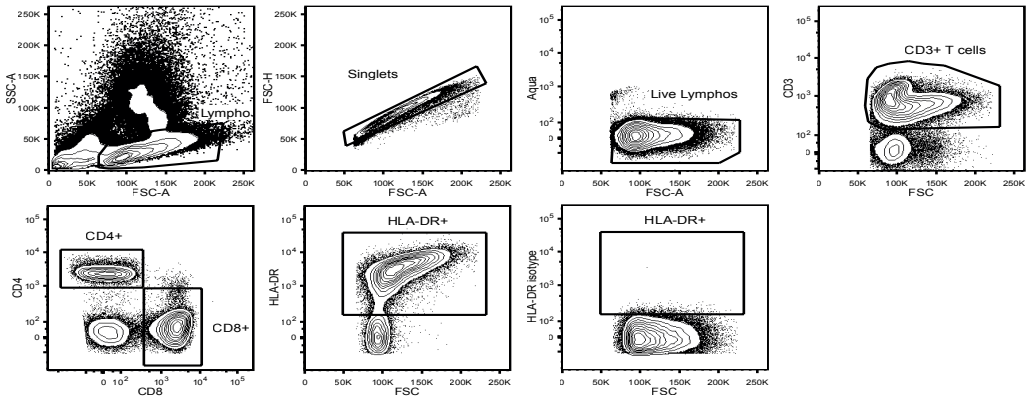
IM subjects	Age (years)	Duration of symptoms (weeks)	HLA-DR+ CD8+ T cells (x10*3 /ml)	NK cells (x10*3 /ml)	EBV DNA copies / ml serum	EBV DNA copies / 10*6 PBMCs	CMV serology
IM01	15	2	1384	341	2178	275	neg
IM02	14	2	270	-	< 100	169	pos
IM03	4	< 1	-	-	170	367	neg
IM04	6	3	843	245	n.d.	9844	neg
IM05	15	2	1432	188	371	88	neg
IM06	3	2	899	530	118	1632	neg
IM07	5	2	4185	1354	410	2910	neg
IM08	15	> 4	252	118	< 100	131	neg
IM09	10	1	2726	1344	2349	6298	neg
IM10	12	2	1083	316	n.d.	50	neg
IM11	2	2	3276	969	8520	2966	pos
IM12	14	< 1	919	212	< 100	1260	pos
IM13	15	< 1	1228	231	2578	31561	neg
IM14	14	> 4	117	160	148	12	pos
IM15	13	< 1	1665	239	721	3811	neg
IM16	7	1	2154	277	382	3922	pos
IM17	5	1	1113	718	4236	9394	pos
IM18	12	1	1602	230	896	5230	neg
IM19	6	< 1	1522	380	13065	16255	neg
IM20	14	< 1	765	307	18473	32157	neg
IM21	15	1	3630	601	4368	347	neg
IM22	3	< 1	816	365	11741	2960	pos

B

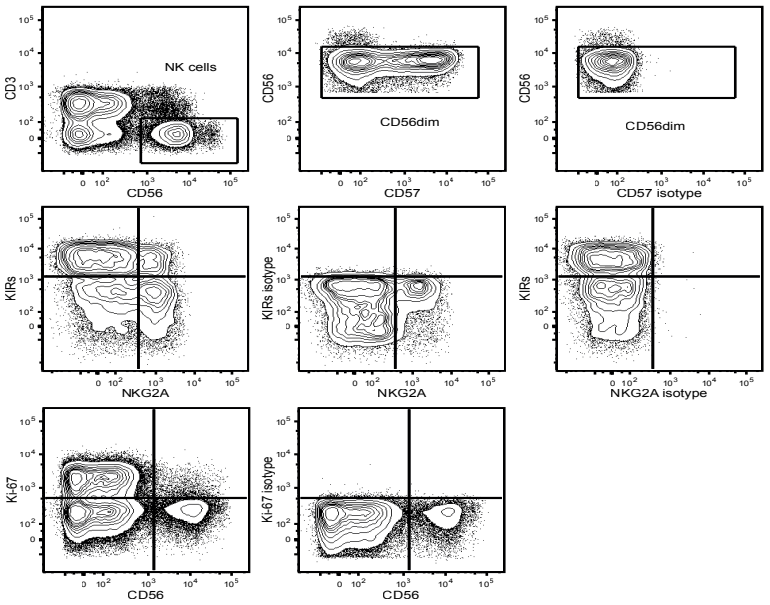
	mean age (years)	median age (years)	age range (years)	median HLA-DR+ CD8+ T cell counts (x10*3 /ml)	median NK cell counts (x10*3 /ml)	CMV sero-postive subjects (%)
IM subjects	9.9	12	2-15	1408	329	31.8
Healthy subjects	11.4	8	2-28	23	185	37.5
IM-like subjects	8.9	9.5	3-15	32	132	58.3

Supplemental Table 1. Patients' characteristics. (**A**) Characteristics of IM patients at acute phase, including age, duration of symptoms, HLA-DR+ CD8+ T and NK cell counts, viral load in plasma and in PBMCs, as well as CMV serology. (**B**) Comparisons between IM patients, IM-like patients and healthy controls.

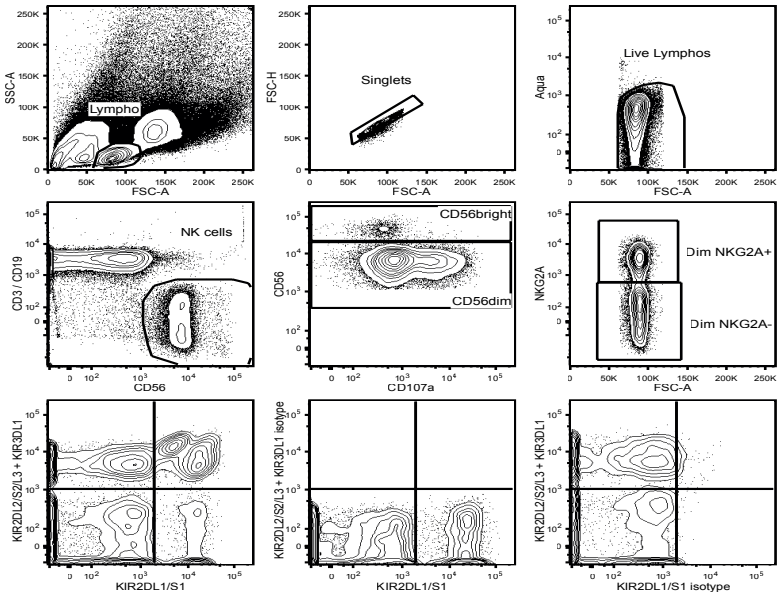
T cell phenotype



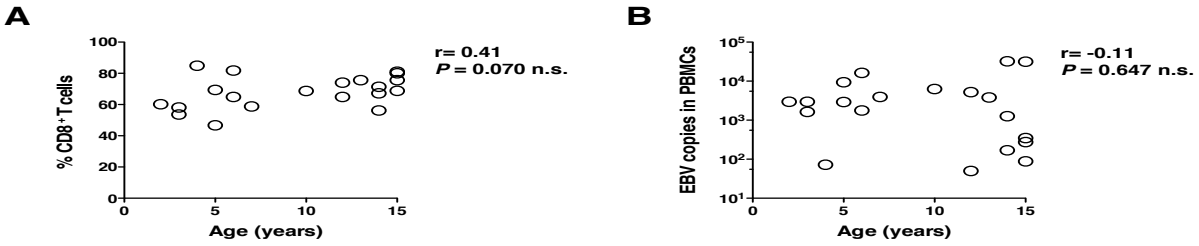
NK cell phenotype



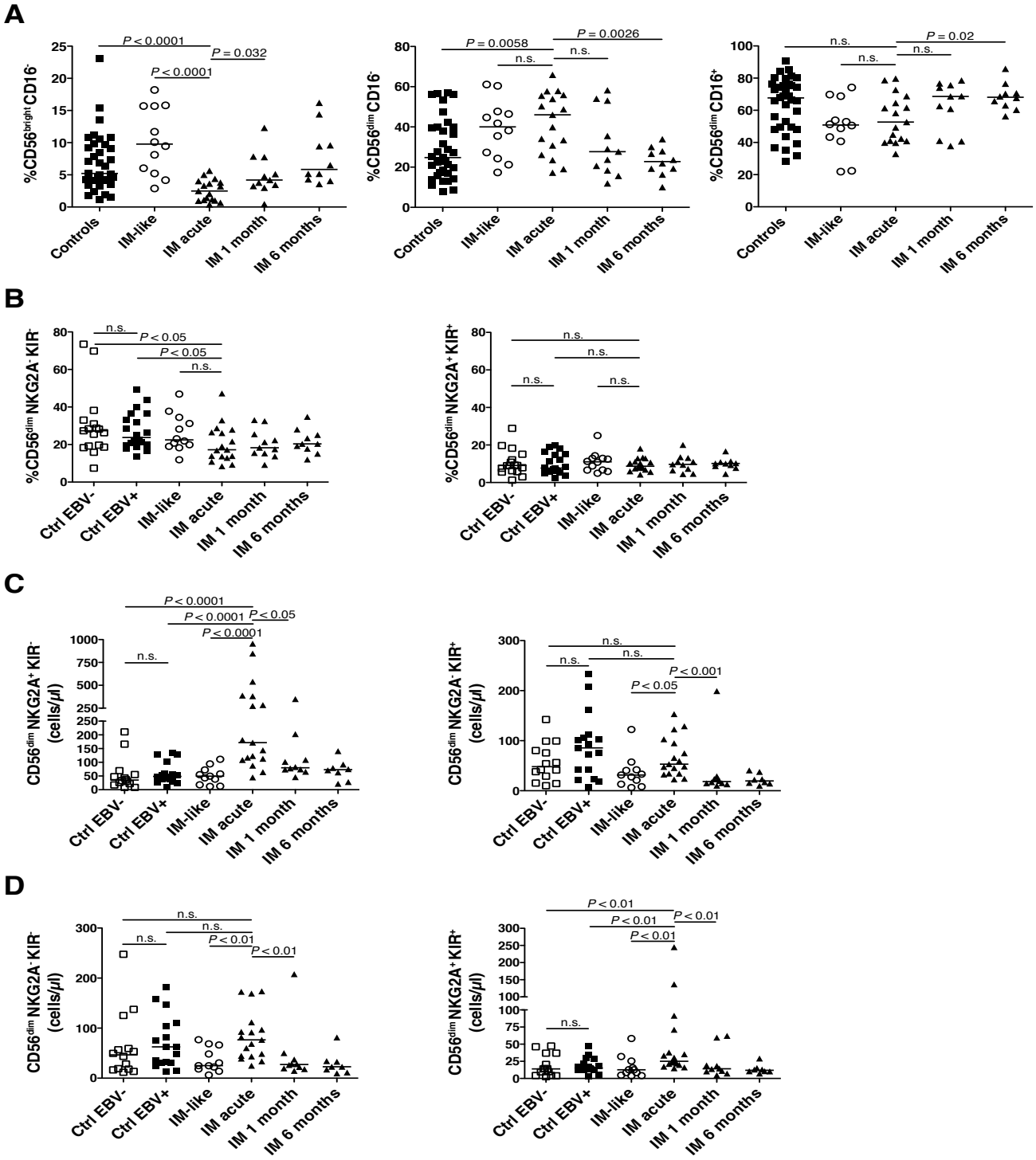
Function assay



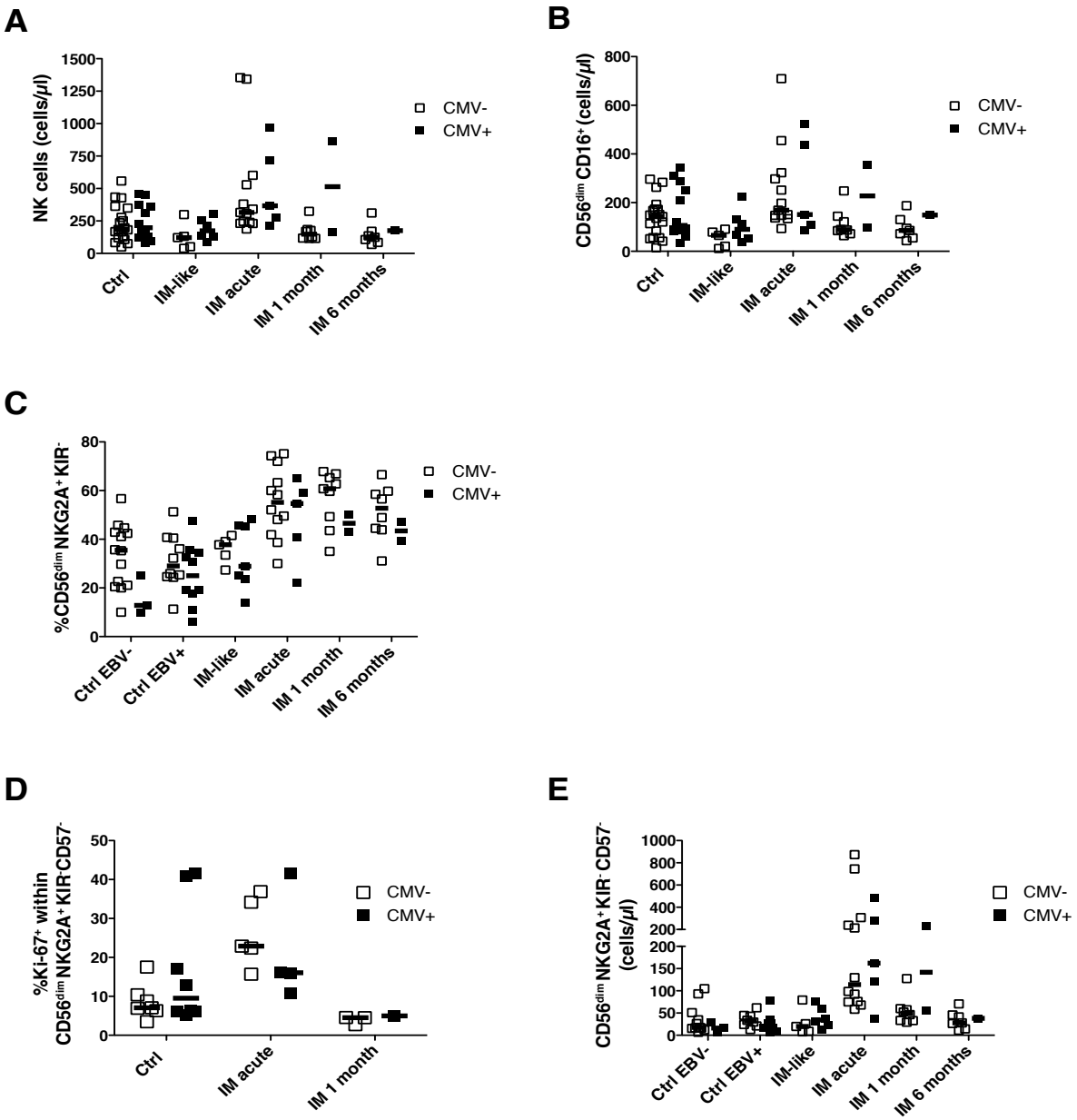
Gating strategies



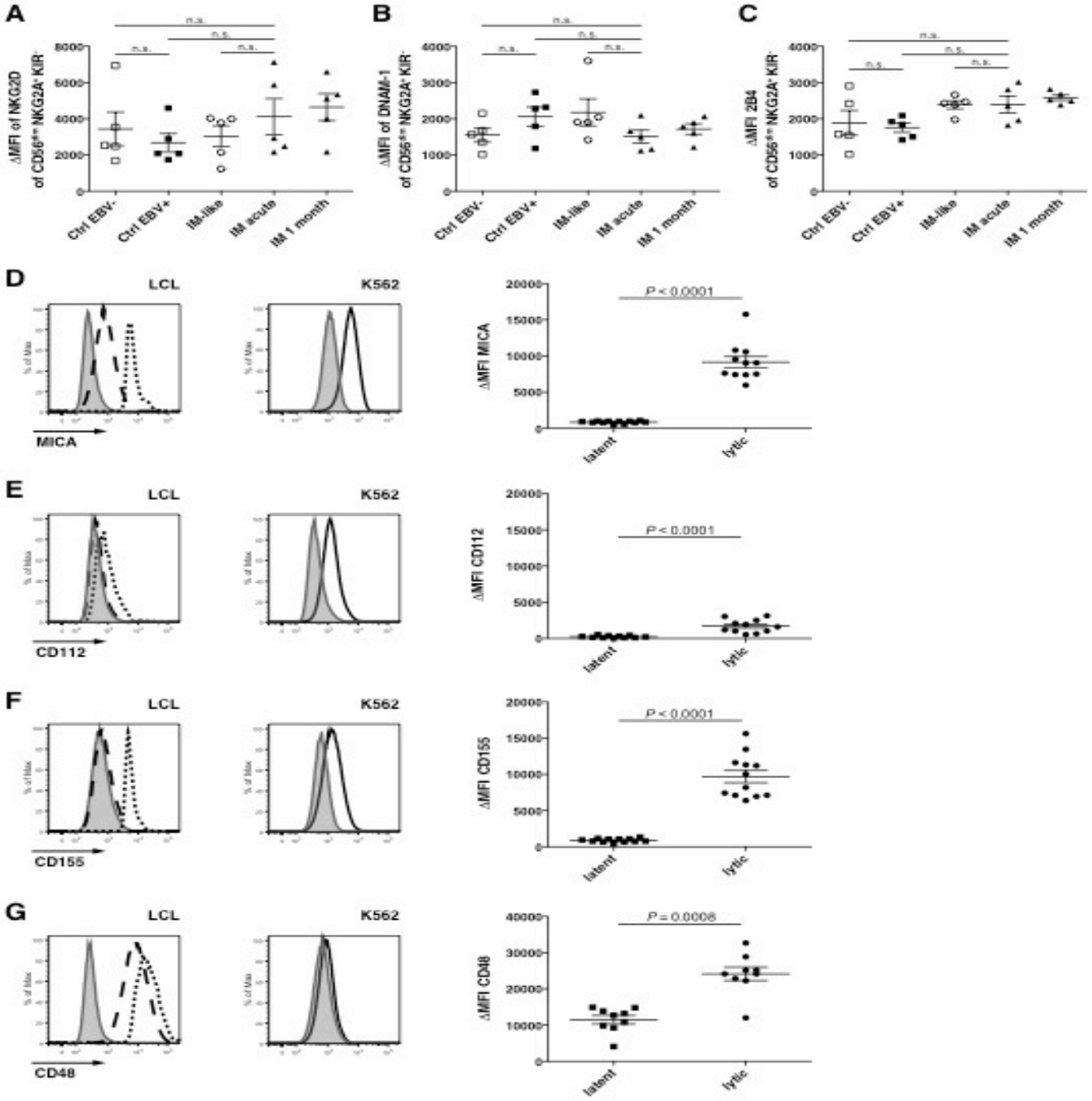
Supplementary Figure 1. Lack of correlation of age and CD8⁺ T cells or EBV DNA loads (**A**) Correlation of age (years) and frequencies of CD8⁺ T cells from acute IM patients. Spearman r = 0.41, P (two-tailed) = 0.070, n=20. (**B**) Correlation of age (years) and EBV DNA loads (copies per 10^6 PBMCs) from acute IM patients. Spearman r = -0.11, P (two-tailed) = 0.647, n=20.



Supplementary Figure 2. Frequencies and cell counts of NK cell subsets during IM. (**A**) Frequencies of $CD56^{bright}$ $CD16^{-}$, $CD56^{dim}$ $CD16^{-}$ and $CD56^{dim}$ $CD16^{+}$ NK cell subsets within the $CD3^{-}$ $CD56^{+}$ NK cell population in healthy controls (n=37), IM-like patients (n=12) and IM acute (n=17), 1 month (n=11) and 6 months (n=10) patients. (**B**) Frequencies of $CD56^{dim}$ $NKG2A^{-}$ KIR^{-} and $CD56^{dim}$ $NKG2A^{+}$ KIR^{+} NK cells in healthy EBV-negative controls (n=17), healthy EBV-positive controls (n=20), IM-like patients (n=12) and IM acute (n=17), 1 month (n=11) and 6 months (n=10) patients. Counts of (**C**) $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} and $CD56^{dim}$ $NKG2A^{-}$ KIR^{+} NK cells and (**D**) $CD56^{dim}$ $NKG2A^{-}$ KIR^{-} and $CD56^{dim}$ $NKG2A^{+}$ KIR^{+} NK cells in healthy EBV-negative controls (n=14), healthy EBV-positive controls (n=17), IM-like patients (n=11) and IM acute (n=17), 1 month (n=10) and 6 months (n=8) patients. Horizontal lines indicate median values of a given symbol. Mann-Whitney tests.



Supplementary Figure 3. No significant differences between CMV+ and CMV- IM patients or CMV+ and CMV- healthy controls. PBMCs from healthy controls, IM-like patients and IM patients at acute phase (IM acute), at 1 (IM 1 month) and 6 months (IM 6 months) were analyzed by flow cytometry. Counts of (**A**) NK cells and (**B**) $CD56^{dim}$ $CD16^{+}$ NK cells in healthy controls (CMV- n=19; CMV+ n=12), IM-like (CMV- n=5; CMV+ n=6) and IM acute (CMV- n=13; CMV+ n=5), 1 month (CMV- n=8; CMV+ n=2) and 6 months (CMV- n=7; CMV+ n=1) patients. (**C**) Frequencies of $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} in healthy EBV-negative controls (CMV- n=14; CMV+ n=3), healthy EBV-positive controls (CMV- n=10; CMV+ n=10), IM-like patients (CMV- n=5; CMV+ n=7) and IM acute (CMV- n=12; CMV+ n=5), 1 month (CMV- n=9; CMV+ n=2) and 6 months (CMV- n=8; CMV+ n=2) patients. (**D**) Frequencies of $Ki\text{-}67^{+}$ cells within the $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} $CD57^{-}$ NK cells in healthy controls (CMV- n=7; CMV+ n=8), IM patients acute (CMV- n=5; CMV+ n=4) and 1 month (CMV- n=3; CMV+ n=1) patients. (**E**) Count of $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} $CD57^{-}$ in healthy EBV-negative controls (CMV- n=11; CMV+ n=3), healthy EBV-positive controls (CMV- n=8; CMV+ n=9), IM-like (CMV- n=5; CMV+ n=6), IM acute (CMV- n=12; CMV+ n=5), 1 month (CMV- n=8; CMV+ n=2) and 6 months (CMV- n=7; CMV+ n=1) patients.



Supplementary Figure 4. Expression of activating NK cell receptors during IM and their ligands. Mean fluorescence intensity on $CD56^{dim}$ $NKG2A^+$ KIR^- NK cells in healthy EBV-negative controls (n=5), healthy EBV-positive controls (n=5), IM-like patients (n=5), IM acute (n=5) and IM 1 month patients (n=5) of NKG2D (**A**), DNAM-1 (**B**) and 2B4 (**C**). Representative histograms of latent ($BZLF1^-$) and lytic ($BZLF1^+$) autologous LCLs (latent LCLs, dashed line; lytic LCLs, dotted line; isotype controls, gray) and K562 cells (solid line), respectively. Expression of the NKG2D ligand MICA (**D**), the DNAM-1 ligands CD112 and CD155, respectively (**E** and **F**) and the 2B4 ligand CD48 (**G**) on latent and lytic autologous LCLs from IM patients (n=4). Cumulative data from 3 independent experiments (**D** - **G**). Horizontal lines indicate mean values with +/- SEM of a given symbol, Mann-Whitney tests.

Supplementary Figure 4 was provided by Anna Lünemann who performed the according experiments, analyzed the data and prepared the figure.

COLLABORATIONS

Oropharyngeal Group A *Streptococcal* Colonization Disrupts Latent Epstein-Barr Virus Infection

Seigo Ueda, Satoshi Uchiyama, Tarik Azzi, Claudine Gysin, Christoph Berger, Michele Bernasconi, Yasuaki Harabuchi, Annelies S. Zinkernagel, and David Nadal

J Infect Dis. 2014 Jan 15;209(2):255-64.

Abstract

Epstein-Barr virus (EBV) infects more than 90% of the human population within the first two decades of life and establishes reversible latent infection in B cells. Which stimuli leads to switching from latent to lytic EBV infection in vivo are still elusive. Group A *Streptococci* (GAS) are a common cause of bacterial pharyngotonsillitis in children and adolescents and colonize tonsil and pharynx of up to 20% of healthy children. Thus, concomitant presence of EBV and GAS in the same individual is frequent. Here, we show that EBV carriers who are colonized with GAS shed EBV particles in higher numbers in their saliva compared to EBV carriers not colonized with GAS. mRNA levels of the master lytic regulatory EBV gene *BZLF1* were more frequently detected in tonsils from EBV-carriers colonized with GAS than from not colonized. Heat-killed GAS, potentially mimicking GAS colonization, elicited lytic EBV in latently infected lymphoblastoid cell lines (LCLs) partially via TLR2 triggering, as did purified GAS peptidoglycan. Thus, EBV may benefit from colonizing GAS that by increasing the EBV load in saliva may contribute to enhancing the viral spread to other hosts.

Own contribution: I performed experiments and contributed to vital reagents and human primary cells.

For detailed information, see attached *manuscript N°3*.

A distinct subpopulation of human NK cells restrict B cell transformation by EBV

Lünemann A, Vanoaica LD, Azzi T, Nadal D and Münz C.

J Immunol. 2013 Nov 15;191(10):4989-95.

Abstract

NK cells constitute the first line of defense against pathogens and transformed cells. They mature in secondary lymphoid organs, including tonsils, where common pathogens, such as EBV, enter the host and potentially imprint differentiating cells, which then patrol the body via the blood stream. Therefore, we set out to characterize a distinct human NK cell population in tonsils that produces high amounts of the immunomodulatory and antiviral cytokine IFN-γ. We found that the tonsilar IFN-γ^{high} NK cell subset is $CD56^{bright}$ $NKG2A^{+}$ $CD94^{+}$ $CD54^{+}$ $CD62L^{-}$, is present in tonsils ex vivo and is more mature than other $CD56^{bright}$ NK cells in tonsils and less mature than other NK cells in blood, shows very low plasticity even after prolonged cytokine stimulation, accumulates in tonsils of EBV carriers, and is able to potently restrict EBV-induced transformation of B cells. Thus, we characterized a distinct and stable IFN-γ^{high} NK cell subpopulation that can specifically restrict malignant transformation of EBV-infected B cells. This subset should be exploited for future development of cell-based therapeutic approaches in EBV-associated malignancies.

Own contribution: I contributed to vital human primary cells.

For detailed information, see attached *manuscript N° 4*.

6. DISCUSSION AND OUTLOOK

NK cells have been originally classified as innate lymphocytes that mediate rapid and unspecific immune responses during viral infections (57). From nearly 40 years of intensive NK cell research, we learned that human NK cells are composed of several subsets, which can preferentially respond to specific viral challenges and exhibit some adaptive immune functions similar to those observed in pathogen-specific T cell responses. Indeed, several longitudinal studies have reported the preferential accumulation of late-differentiated $CD56^{dim}$ $NKG2C^{+}$ (102, 103) or $CD56^{dim}$ KIR^{+} (136) NK cell subsets.

In this thesis, we longitudinally investigated the responses of NK cell subsets in peripheral blood from pediatric patients with acute primary symptomatic EBV infection or infectious mononucleosis (IM). We reported for the first time that 1) EBV-associated IM elicits the expansion and long-lasting accumulation of only one specific NK cell subset as observed during other acute viral infections; 2) however, IM drives the unique and specific proliferation of an early-differentiated $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} NK cell subset, which has never been described in other infectious or immunological diseases; 3) this particular NK cell subset can directly recognize and target EBV-infected cells with lytic replication, but not EBV transformed B cells or LCLs. This suggests that a rapid response from $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} NK cells might limit early lytic EBV replication, and therefore consistently decrease the numbers of EBV-infected B cells before the development of the adaptive T cell responses; 4) the frequencies and absolute numbers of these NK cells consistently decrease with increasing age, suggesting that the early innate NK cell-mediated immune control of EBV lytic replication might be stronger during early childhood. This hypothesis would fit with the age-related predisposition to infectious mononucleosis.

The preferential proliferation of $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} NK cells during acute IM, as demonstrated with *ex vivo* staining for the proliferation marker Ki-67, seems to be directly triggered by cell-contact with EBV-infected B cells, and is not a consequence of bystander activation mediated by proinflammatory cytokines. This is suggested by several observations: firstly, other acute viral infections with high levels of cytokines elicit expansion of other NK cell subsets; secondly, our control group of age-matched pediatric patients with IM-like symptoms does not exhibit such NK cell subset expansions; thirdly, the $CD56^{bright}$ precursor subset, with its well established superior proliferative responsiveness upon cytokine stimulation, does not display increased proliferation during acute IM. Moreover, our study

suggests that the proliferation of this NK subset partially depends on expression of EBV lytic antigens, as shown by *in vitro* infection of cord blood mononuclear cells (CBMCs) with the recombinant wildtype EBV compared to the recombinant BZLF1-KO EBV, which does not express any lytic antigens. These findings are in good agreement with the recently published work by Chijioke and colleagues on EBV infection in mice with human immune system (123). In this model CD16+ NKG2A+ KIR- NK cells accumulate upon EBV infection and mediates immune protection exclusively against the wildtype EBV strain expressing lytic antigens, but not against a BZLF1-knockout virus with defective lytic replication. This further support the role of early-differentiated NK cells during EBV infection in humans.

Furthermore, we could also demonstrate that the proliferating $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} $CD57^{-}$ NK cells terminally differentiate into the $CD57^{+}$ subset, and not into the KIR^{+} subset, during the first month of IM. This represents the first *in vivo* description of NK cell terminal differentiation within the $NKG2A^{+}$ NK cell subset. Interestingly, convalescent IM patients still exhibited at the first month visit post-IM increased numbers of $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} $CD57^{+}$ NK cells compared to EBV-naïve uninfected controls. The maturation marker CD57 has been postulated to be a marker of human memory NK cells, which might be up-regulated on expanding NK cells during viral infections (101). Thus, if the adaptive features of mouse NK cells turn to be conserved in humans, a fraction of the $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} $CD57^{+}$ NK cell subset could actually represent EBV-specific memory NK cells, which have been primed during infectious mononucleosis.

Nevertheless, the exact mechanisms behind the specific recognition of EBV-infected B cells by $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} NK cells remain unclear, but need to be further addressed in detail. Future studies should determine which activating NK cell ligands are up-regulated on EBV-infected cells, therefore triggering NK cell activation, and how lytic EBV infection leads to the up-regulation of these ligands. Stress-associated ligands such as NKG2DL have been reported to get up-regulated upon induction of the DNA damage response and cytosolic DNA recognition (156). Although EBV packages its DNA into the viral capsid in the nucleus during lytic replication, some of it might become accessible during capsid transit through the cytosol and trigger this pathway of NKG2D ligand up-regulation. Indeed, the triggering of lytic reactivation in B cell lines latently infected by EBV elicits a cellular DNA damage response with activation of the ataxia telangiectasia-mutated (ATM) signal transduction pathway (157).

On the other hand, the presence of the inhibitory receptor NKG2A, and not of the KIR, on virtually proliferating NK cells during IM is not clear. This might be explained by the

preferential proliferation of NKG2A+ NK cells or the selective up-regulation of this receptor on NKG2A- proliferating NK cells. Our degranulation data against AKBM however rather support the former hypothesis, since NKG2A+ NK cells preferentially recognize B cells with lytic reactivation. The selective proliferation advantage of NKG2A+ NK cells over KIR+ NK cells during acute IM might be due to an higher inhibition threshold for the former receptor, allowing this NK cell subset to get activated and proliferate faster in the inflammation environment in IM individuals. Additionally, the potential role of EBV-specific peptides in the modulation of interactions between HLA-E and NKG2A (152, 158, 159), as well as between HLA-C and activating KIRs (128), also needs to be clarified.

Despite efficient innate and adaptive immune responses, this herpesvirus manages to establish latency in memory B cells in a very successful way since it persistently infects the vast majority of the adult population worldwide. Some of these infected B cells exhibit periodic viral reactivation mainly in the tonsils and viral shedding is observed in saliva from convalescent IM patients (31, 43)as well as from some EBV-seropositive children with and without tonsilar enlargement (160). Such persistent EBV lytic replication in the tonsils might indicate an impaired immune control at this site. Indeed, several viral proteins of the lytic cycle are known to provide EBV with immune evasion mechanisms (161). However, the reason why some EBV-seropositive children exhibit an increased shedding in saliva and therefore increased EBV reactivation in tonsils is unclear. Co-infections with other pathogens or commensals in the tonsils such as group A streptococci (GAS) might influence the switch between latent and lytic EBV infection. Indeed, we could demonstrate in collaboration with Annelies Zinkernagel's lab (Division of Infectious Diseases, University Hospital of Zurich) that the amount of viral shedding in saliva as well as the frequency of tonsils positive for BZLF1 (master lytic regulatory EBV gene) mRNA expression was significantly higher in EBV carriers with concomitant GAS colonization compared to without GAS colonization (attached manuscript N°3; (162)). Exposure of EBV latently infected LCL to heat-killed GAS, potentially mimicking GAS colonization, could trigger the lytic reactivation of these cells as measured by BZLF1 mRNA expression. This effect was shown to be partially TLR2-dependent, since exposure to purified GAS peptidoglycan (TLR2 ligand) also elicits BZLF1 up-regulation on LCLs, which was partially inhibited in TLR2 blocking experiments.

Most of this work focused on the NK cell-mediated cytotoxicity against EBV-infected B cells with lytic replication by a newly described functional subset. On the other hand, we could show that the early-differentiated NK cell subset, as well as other NK cell subsets from peripheral blood, cannot target the EBV-transformed B cell or LCL in vitro. This is probably

due to the EBV-mediated up-regulation of the inhibitory ligands HLA class I and HLA-E, which protects the LCL from NK cell killing. However, the immune control of transformed B cells in vivo is probably mainly mediated by EBV-specific T cells during primary infection of immunocompetent individuals. Accordingly, post-transplant lymphoproliferative disease (or PTLD) in immunocompromised patients can be cured by infusion of EBV-specific in vitro expanded T cell lines (45). NK cells might rather play a role in delaying the viral transformation process of the infected B cells in the tonsils, the portal of entry of EBV, until the development of EBV-specific T cell responses. Indeed, tonsilar NK cells restrict B cell transformation more efficiently than peripheral blood NK cells. This process depends on IFN-γ production by NK cells after their activation by DCs-secreted IL-12 (120). IFN-γ is able to curb the B cell transformation in vitro by delaying the expression of EBV main oncogene LMP1. Notably, this restriction is only possible during the first four days of EBV infection and treatment with recombinant IFN-γ at latter time points during transformation does not influence anymore the outgrowth of LCLs. Recently, Lünemann et al. could show in collaboration with our lab that a newly characterized tonsilar NK cell subset mediates this restriction via a superior secretion of IFN-γ (attached manuscript N°4; (163)) and that this subset accumulates in the tonsils, but not in peripheral blood, of EBV-seropositive children. Interestingly, these NK cells are $CD56^{bright}$ $NKG2A^{+}$ and mainly KIR^{-} and might actually be the direct precursors of $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} NK cells that are preferentially proliferating in peripheral blood of acute IM patients. Future studies should analyze the phenotype and function of NK cell subsets in acutely infected tonsils from IM patients, although emergency tonsillectomies are only performed on IM patients with life-threatening upper airway obstruction and are therefore extremely rare (164).

Age-related manifestations of primary infections have been described with several viruses such as EBV, CMV, human immunodeficiency virus (HIV) and varicella zoster virus (VZV). Indeed, primary infections with these viruses in early childhood are often asymptomatic or less severe compared to primary infections in older individuals such as teenagers and adults. In this thesis, we could not observe any differences in age-dependent NK cell responses against EBV transformed B cells or any differences in age-dependent expression level of NK cell activating receptors known to be involved in recognition of EBV infected cells. However, we report major changes in the distribution of blood NK cell subsets according to age. Indeed, $CD56^{bright}$ $CD16^{-}$ and $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} NK cells, which are considered immature or early-differentiated, are significantly more prominent in young children. This age-dependent maturation of the NK cell compartment is probably driven by

environmental stimuli such as pathogen encounters during the lifetime of the individual. A picture is now emerging in which late-differentiated NK cell subsets predominate in peripheral blood later in life time, as also suggested by the specific temporal reconstitution of the NK cell subsets in hematopoietic stem cell (HSC) transplanted patients (149-151)and in mice with humanized immune system (100).Our data indicate that decreased numbers of $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} NK cells in peripheral blood of teenagers or adults might lead to an impaired early immune control of the EBV lytic replication, leading to increased numbers of EBV-infected B cells with lytic antigens, which could trigger the extensive IM-associated immune responses with the characteristic expansion of $CD8^{+}$ T cells specific for EBV lytic epitopes. This hypothesis has been supported recently in a study of EBV infection in humanized mice (123). In these mice, more than 70% of human NK cells are early-differentiated and exhibit an $NKG2A^{+}$ KIR^{-} phenotype such as observed in children younger than 5 years. Depletion of human NK cells before EBV infection leads to decreased immune control of EBV-infected B cells (increased viral loads and increased tumor formation) and enhance IM symptoms (increased $CD8^{+}$ T cell frequency, increased level of IFN-γ and increased spleen size) compared to non-depleted infected animals. Cytokine treatment of reconstituted mice leads to NK cell differentiation and increased expression of KIR molecules, resembling NK cells in individuals older than 5 years. However these late-differentiated NK cells control EBV less well in vivo, suggesting that older individuals might display a quantitatively impaired NK cell-mediated immune control of EBV compared to young children. Such a hypothesis could in the end only be verified prospectively by analyzing the NK cell compartment in peripheral blood of EBV-negative individuals before and after the development of IM. Such an approach has been proven feasible with EBV-negative university students (17)and would provide a fantastic tool to better understand and study innate immune responses during primary EBV infection.

EBV infection is associated with several malignancies of the B cell and epithelial cell origin (see table 1) both in immunocompetent and immunocompromised individuals, and might also be linked to autoimmune diseases (165). The role of NK cell subsets in the immune control of EBV-positive tumors is unclear and NK cell responses in patients with such pathologies have not been investigated. In this thesis, we could show that $CD56^{dim}$ $NKG2A^{+}$ KIR^{-} NK cells preferentially react to EBV-infected B cells with expression of lytic antigens, and not with the transformation-associated latent antigens. This might indicate that this NK cell subset can only target EBV-positive tumors expressing lytic antigens. Interestingly, patients suffering from EBV-positive tumors such as Burkitt lymphoma (166),

Hodgkin lymphoma (167, 168)and nasopharyngeal carcinoma (169)exhibit raised titers of antibodies specific for the late lytic viral capsid antigen (VCA). The expansion of these antibody responses probably follows the availability of their respective antigens, indicating that some of the EBV-positive tumor cells might express lytic antigen and could therefore be targeted by NK cell-mediated immunotherapy.

7. LITERATURE

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10. MANUSCRIPTS

1) **Innate immune responses against Epstein Barr virus infection (review)**

2) **Role for early-differentiated natural killer cells in infectious mononucleosis**

3) **Oropharyngeal Group A *Streptococcal* Colonization Disrupts Latent Epstein-Barr Virus Infection**

4) **A distinct subpopulation of human NK cells restrict B cell transformation by EBV**

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JLB

Review

Innate immune responses against Epstein Barr virus infection

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ABSTRACT

EBV persists life-long in >95% of the human adult population. Whereas it is perfectly immune-controlled in most infected individuals, a minority develops EBV-associated diseases, primarily malignancies of B cell and epithelial cell origin. In recent years, it has become apparent that the course of primary infection determines part of the risk to develop EBV-associated diseases. Particularly, the primary symptomatic EBV infection or IM, which is caused by exaggerated T cell responses, resulting in EBV-induced lymphocytosis, predisposes for EBV-associated diseases. The role of innate immunity in the development of IM remains unknown. Therefore, it is important to understand how the innate immune response to this virus differs between symptomatic and asymptomatic primary EBV infection. Furthermore, the efficiency of innate immune compartments might determine the outcome of primary infection and could explain why some individuals are susceptible to IM. We will discuss these aspects in this review with a focus on intrinsic immunity in EBV-infected B cells, as well as innate immune responses by DCs and NK cells, which constitute promising immune compartments for the understanding of early immune control against EBV and potential targets for EBV-specific immunotherapies. *J. Leukoc. Biol.* 94: 000–000; 2013.

EBV INFECTION AND ASSOCIATED DISEASES

EBV belongs to the γ subfamily of herpesviruses and was first identified in cultured lymphoma cells derived from an African BL patient [1]. The global seroprevalence is very high, suggesting that 95% of adults worldwide are infected with EBV [2]. In contrast to diseases that are associated with persistent infection [3], primary infection with EBV usually occurs in childhood and remains asymptomatic during the acute phase, whereas in adolescents and adults, primary infection is symptomatic in >70% of cases [4] and presents as IM. IM is a self-limiting disease with characteristic clinical symptoms of pharyngitis, fever, fatigue, cervical lymphadenopathy, hepatosplenomegaly, and T cell lymphocytosis in the peripheral blood. Although EBV persists in infected individuals, both after asymptomatic primary infection and IM, it is usually immune-controlled but without viral clearance for the rest of the host's life [5]. However, during the first 5 years after resolving IM, the risk for EBV-positive Hodgkin lymphoma is increased up to five times [6, 7], and a history of IM increases the risk to develop multiple sclerosis by twofold [8, 9]. In rare cases, primary infection is fatal and is the most common cause of infection-associated HLH [10]. Therefore, symptomatic primary EBV infection predisposes for diseases associated with this virus.

EBV is associated with several malignancies of B cell origin, the primary target of EBV. Almost 100% of endemic BL is positive for EBV. Because of the invariant presence of *c-myc* translocations in BL, however, the exact role of EBV in the transforming process is still not defined clearly [11, 12]. Similarly, classical Hodgkin lymphoma, most frequently of the mixed cellularity subtype, is associated with EBV in 40% of cases. However, as in BL, the culprit for tumorigenesis might not be EBV alone, as this disease is multifactorial [13]. The impact of the host immune response on the control of EBV becomes evident in immunosuppressed patients and patients with primary immunodeficiencies. PTLDs comprise a variety of lymphoid disorders, ranging from polymorphic lymphoid hyperplasia to malignant lymphoma, and are associated mostly with EBV infection [14]. The risk for EBV-associated PTLDs correlates with the degree of immunosuppression, such that the highest incidence is found in solid organ transplant patients with the most intense immunosuppressive regimes. Moreover, virtually all AIDS-related CNS lymphomas and leiomyosarcomas are associated with EBV [15, 16]. Patients suffering from XLP disease are exclusively fragile to primary EBV infection but not any other pathogens and develop fulminant and some-

Abbreviations: BGLF5=Bam H1 G leftward open reading frame 5, BL=Burkitt's lymphoma, BZLF1=Bam H1 Z leftward open reading frame 1, cDC=conventional DC, EBER=EBV-encoded, small, noncoding nuclear RNA, HLH=hemophagocytic lymphohistiocytosis, IM=infectious mononucleosis, KIR=killer cell Ig-like receptor, LMP-1=latent membrane protein 1, pDC=plasmacytoid DC, PTLD=post-transplant lymphoproliferative disorder, XLP=X-linked lymphoproliferative

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times lethal IM, as well as B cell lymphomas, after severe primary EBV infection. The disease-causing mutations lead to an impairment of T and NK cells to target EBV-infected cells [17–20]. In rare cases, EBV infects T or NK cells, leading to the development of T cell [21] and NK cell lymphomas [22]. EBV is also associated with nonlymphoid malignancies, such as nasopharyngeal carcinoma [23] and EBV-positive gastric carcinoma [24]. In both entities, EBV infection seems to occur before malignant transformation, as EBV is monoclonal in tumor cells [25, 26]. The exact contribution of EBV in this plethora of associated malignancies is still ill-defined, but the presence of EBV gene products capable of inducing cellular transformation on their own, infection of every tumor cell, and epidemiological evidence supports the notion of a pathogenic role for EBV.

EBV-ASSOCIATED MOLECULAR PATTERNS THAT ELICIT IMMUNE ACTIVATION

The formidable immune control that keeps EBV in check for >50 years in most infected individuals requires priming of protective T cell responses [5]. For this to occur, EBV-associated molecular patterns need to be recognized by the immune system, probably directly by APCs for optimal T cell priming [27]. APCs carry a number of PRRs, including TLRs, nucleotide-binding oligomerization-like receptors, retinoic acid-inducible gene-like receptors, and C-type lectin-like receptors [28]. Of these, TLR3 and TLR9 have been implicated in sensing of EBV and might complement each other for the recognition of this virus by cDCs and pDCs, respectively (**Fig. 1**). In addition, monocytes, upon detection of EBV via TLR2, secrete cytokines and chemokines [29–31], whereas TLR7 signaling has been reported to be modulated by EBV [32, 33]. Notably, B cells, the primary target of EBV, also express TLR3 and TLR9 [34]. In contrast to mice, TLR3 and TLR9 are on separate DC subsets in humans. TLR3 is expressed primarily by cDCs, whereas TLR9 is restricted to pDCs [35]. Therefore, both major human DC subsets could detect different cues during EBV infection, whereas the primary target cell of EBV is furnished with both receptors.

TLR3 recognizes dsRNA in endosomal compartments of human cDCs. EBERs have been described to form stem-loop structures, and the resulting RNA was reported to bind to TLR3 [36]. EBERs were found to be released from EBV-infected cells in complex with the La protein, a frequent autoantigen in systemic lupus erythematosus and Sjögren's syndrome. Sera of patients with IM, chronic active EBV infection, and EBV-associated HLH, diseases whose pathology might be mainly mediated by the release of large amounts of proinflammatory cytokines, were demonstrated to contain high concentrations of EBERs. Indeed, these sera elicited TLR3-dependent cytokine production and DC maturation in vitro. These data suggest that dsRNA structures of EBERs might stimulate cDCs via TLR3 to allow them to prime protective EBV-specific T cell responses.

In contrast, it is much less clear to which extend human pDCs can initiate T cell responses. However, they seem to be

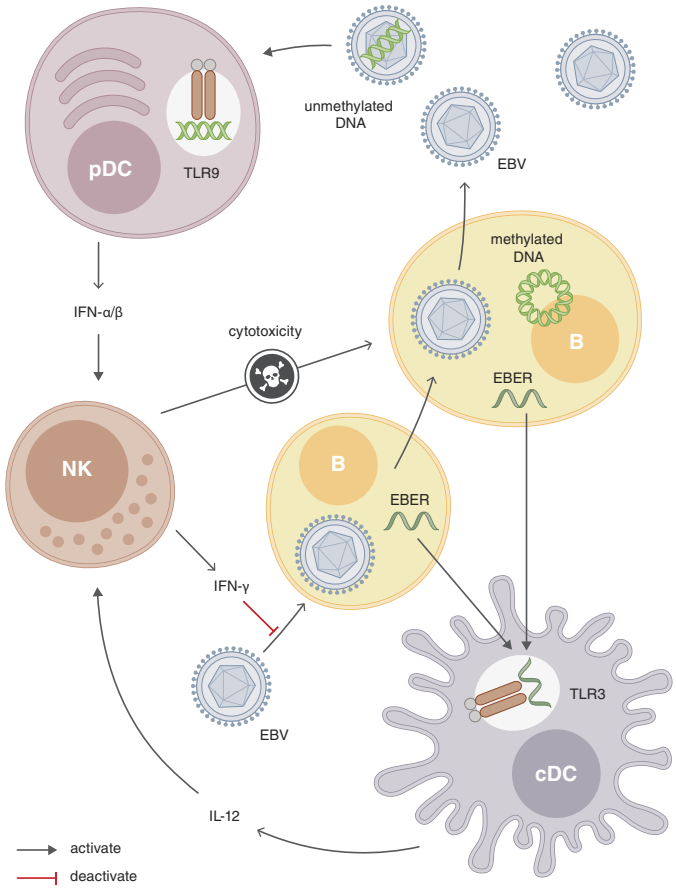

Figure 1. Innate immune recognition via identified PRR ligands and innate restriction of EBV. EBV seems to activate pDCs and cDCs with its unmethylated viral DNA and EBER, respectively. These pathogen-associated patterns engage TLR9 on pDCs and TLR3 on cDCs. NK cells, which can be activated by pDCs and cDCs to produce IFN-γ in response mainly to IL-12 and increase cytotoxicity upon encountering IFN-α/β, can restrict EBV infection. NK cells prevent B cell transformation via IFN-γ and kill lytically EBV-replicating cells.

able to recognize EBV DNA via the endosomal receptor TLR9 [31, 37], which recognizes unmethylated CpG DNA motifs. Linear dsDNA in EBV particles is unmethylated, but circularizes after cellular infection and then gets hypermethylated successively [38], suggesting that especially noncell-associated virus-encapsulated EBV DNA should be immunostimulatory via TLR9. In addition to pDCs, TLR9 is expressed prominently in human B cells, and its stimulation seems to facilitate B cell transformation by EBV [39]. Once infection, however, has occurred, EBV seems to down-regulate TLR9-mediated signaling [33]. The LMP-1 of EBV down-regulates TLR9 signaling during latent infection [40], whereas the lytic EBV antigen BGLF5 down-regulates TLR9 during lytic replication [41] (**Fig. 2**). In addition, even UV-inactivated EBV has been reported to down-regulate TLR9 [32]. In this fashion, EBV possibly protects itself from cell death-inducing and lytic reactivation-inhibiting functions of TLR9 signaling [42, 43]. Thus, EBV, possibly primarily the virus-encapsulated DNA, can be sensed by TLR9 in pDCs and B cells, but the virus protects itself from innate im-

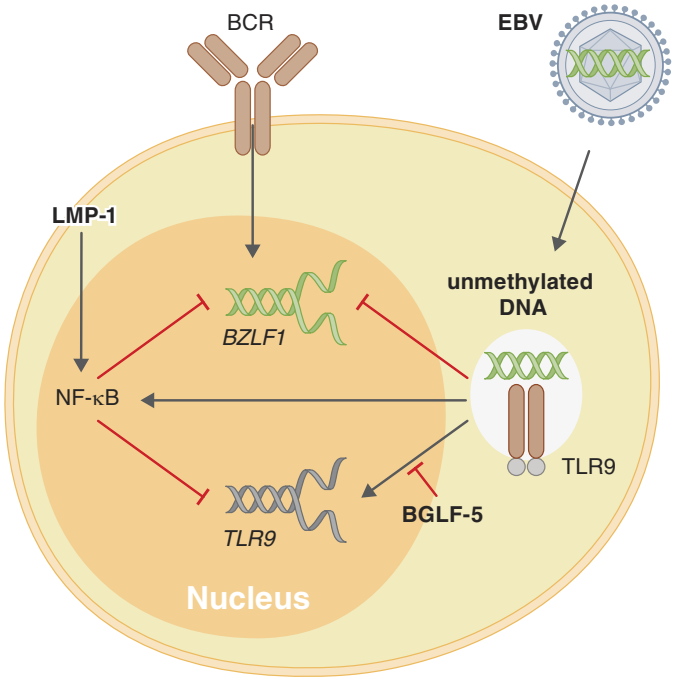

Figure 2. Known interactions between EBV and innate immunity elements in B cells. The published, studied interactions between EBV and innate immunity elements in B cells are virtually limited to TLR9. The unmethylated DNA of EBV triggers TLR9, which suppresses lytic reactivation of EBV by suppressing transcription of the master regulatory lytic gene of EBV, *BZLF1*, and activates NF-κB, which also suppresses *BZLF1* transcription but to a lower extent, whereas triggering the BCR activates *BZLF1* and switches infection to the lytic cycle. Conversely, LMP-1 of EBV suppresses transcription of the *TLR9* gene via NF-κB activation, and the lytic-phase of EBV protein, BGLF5, contributes to down-regulating TLR9 levels by degrading RNA.

mune signaling via this receptor by down-regulating it during latent and lytic infection.

DCs IN INNATE IMMUNE CONTROL OF EBV INFECTION

This TLR-mediated recognition seems to alarm the immune system to EBV infection and matures DCs to form a first barrier against it. Particularly, pDCs have been described as superior sources of the antiviral type I IFN (IFN-α/β) cytokines [44]. In a PBMC transfer model into immunocompromised SCID mice, this IFN-α production by pDCs seemed to be necessary to control EBV infection [37]. PBMCs depleted for pDCs supported disseminated EBV infection, whereas pDC supplementation of PBMCs was able to control infection better after EBV challenge in vivo. The pDC activation by EBV was TLR9-mediated in this system and led to enhanced NK and T cell activation (Fig. 1). As the PBMCs in this model were derived from EBV-seropositive individuals, however, pDCs most likely only supported secondary T cell responses that had been primed in the PBMC donor. It is likely that these secondary responses supported the observed, lasting immune control over EBV infection for 1–2 months, as IFN-α has only been described to block EBV infection of B cells for the first 24 h after infection [45]. The TLR9-mediated detection of EBV by pDCs leading to IFN-α production has been confirmed in independent studies [31, 46]. Thus, pDCs produce the antiviral cytokine IFN-α in response to EBV infection, which restricts the virus initially as well as stimulates other innate immune cells and promotes the development of adaptive immune responses.

These adaptive, particularly T cell-mediated immune responses are, however, most likely not primed by pDCs and also not by EBV-infected B cells [47, 48]. In contrast, inflammatory DCs, modeled in humans by differentiation from monocytes, are able to prime EBV-specific T cell responses, which in turn, can control B cell transformation by EBV in vitro [47]. To fulfill this priming function, monocyte-derived DCs are most likely activated by EBV via TLR2 or TLR3 [31, 36]. However, only TLR3-mediated DC maturation has been reported to lead to the up-regulation of costimulatory molecules and antigen presentation [36], whereas TLR2-mediated monocyte stimulation was shown to lead to the production of MCP1 and the immune-suppressive cytokine IL-10 [31]. In addition, the molecular identity of a TLR2 agonist in EBV is still unknown. Thus, cDCs or inflammatory DCs are probably involved in the priming of protective T cell responses against EBV.

The major human DC subsets—cDCs, inflammatory, and pDCs—are activated, most likely, by EBV after uptake of viral particles or fragments of EBV-infected B cells. Indeed, human monocyte-derived DCs can cross-present EBV-infected B cell fragments to stimulate $CD4^+$ and $CD8^+$ T cells [49, 50]. Moreover, pDCs get activated by purified viral DNA [31]. However, the possibility still exists that a small subset of inflammatory DCs and pDCs is also infected directly by EBV. Along these lines, entry of the virus and access of the viral DNA to the nucleus have been, at least, demonstrated for monocyte-derived DCs and pDCs [46, 51]. However, viral antigen expression has, so far, not been demonstrated convincingly in DCs. Thus, DCs detect, most likely, virus particles directly or products of EBV-infected cells to mount innate immune responses and initiate adaptive immune control of EBV.

NK CELLS IN EBV RESTRICTION

NK cells are known to be important players involved in immune control of cancer and virus-infected cells [52]. In the case of EBV, observational data in humans support a role for human NK cells during innate immune responses to this virus. During symptomatic, primary EBV infection, IM, numbers, and frequency of NK cells in peripheral blood are increased [4, 53–56]. However, one cohort study reported a direct correlation of NK cell count and blood EBV DNA load [4], whereas another smaller study showed an inverse correlation of NK cell frequency and blood EBV DNA load, as well as an inverse correlation of NK cell count and severity of symptoms [55], suggestive of a direct, beneficial contribution by human NK cells in the latter report. However, during viral infections, distinct, terminally differentiated $NKG2C^+KIR^+$ NK cell subsets have been found to be expanded. This was observed during acute and persistent infection with human CMV, hantavirus, or chi-

kungunya virus [57–59]. Therefore, interrogating the bulk NK cell response might not necessarily identify the potentially protective impact of these cells during primary EBV infection.

The antitumoral role of NK cells was highlighted by a recent report of a patient with selective NK cell deficiency, who developed a rare EBV-positive smooth muscle tumor [60]. In another case report, EBV-associated lymphoproliferative disease occurred in a patient with NK cell deficiency [61]. On the molecular level of pathogenesis, mechanistic insight was gained from examining defective immune responses in XLP disease, characterized by extreme susceptibility to EBV [62–64]: blockade of the surface molecules 2B4 and NK-T-B antigen (NTB-A), expressed on normal NK cells and $CD8^+$ T cells, was shown to decrease NK cell cytotoxicity to EBV-positive target cells [17, 18], and both molecules act as inhibitory receptors rather than activating in XLP patients, as a result of mutations in the gene encoding the adaptor protein SLAM-associated protein [65], such that NK and $CD8^+$ T cells are no more able to lyse EBV-infected B cells or respond specifically by cytokine production [17–20]. Furthermore, natural cytotoxicity receptors (i.e., NKp30, NKp44, and NKp46) and NKG2D, all activating NK cell receptors, have been described to be involved in cytotoxic recognition of EBV-positive cell lines [66]. The activating KIR, KIR2DS1, might also play a part in NK cell recognition of EBV-infected cells, as only EBV, but not other herpes viruses, up-regulate ligands for KIR2DS1 upon infection, and the blocking of this receptor diminishes lysis of EBV-positive target cells to some extent [67]. Interestingly, lytic EBV infection compared with latent infection renders target cells more susceptible to NK cell killing (Fig. 1), probably as a result of down-regulation of MHC-I molecules and increased expression of UL16-binding protein 1 and CD112, ligands for the activating receptors NKG2D and DNAX accessory molecule-1 (DNAM-1), respectively [68]. Finally, NK cells might function tissue-specifically, as it has been shown that tonsillar NK cells restrict B cell transformation more efficiently than peripheral blood NK cells. This process depends on IFN-γ production by NK cells after their activation by IL-12, produced by DCs [69]. In summary, there is growing evidence that human NK cells are involved in the immune response against EBV in vivo. However, the receptor-ligand interactions that are important for NK cell-mediated immune control of EBV and which infection program is restricted by NK cells in vivo still need to be better defined.

CONCLUSIONS AND OUTLOOK

EBV is one of the examples of a persistent human pathogen that can be controlled by the immune system for extended time periods, suggesting that the respective protective immune responses have been primed to cope optimally with this lifelong challenge. Thus, this herpesvirus can teach us how protective immunity has to be shaped and which innate immune compartments must be engaged to achieve this lasting immune control. This knowledge might lead us to the development of novel vaccine adjuvants that stimulate innate immune responses and therefore, promote long-lasting protection against other chronic infectious diseases. Along these lines, we have learned that EBV probably provides different PAMPs that engage pDCs and cDCs for optimal T cell priming. Furthermore, innate lymphocyte compartments, such as NK cells, might have been selected during evolution to restrict the early phases of EBV infection to gain time for the adaptive immune response to build its comprehensive immune control. Finally, EBV has hijacked part of the intrinsic immune recognition mechanisms in B cells to improve infection and regulate reactivation. However, the fact that IM patients already exhibit strong adaptive immune responses when they seek medical care complicates the study of early events during acute EBV infection. Thus, to learn more about EBV-specific innate immunity, experimental systems that allow the dissection of human immune responses in vivo have to be developed further. With the advent of humanized mouse models that support reconstitution of human immune system compartments, such systems become available, which will not only allow dissection of innate immune responses to EBV in more detail but also provide a platform to test vaccine candidates, whose protective efficiency can then be challenged in vivo.

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DISCLOSURES

The authors declare no conflict of interest with the discussed topics.

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Regular Article

IMMUNOBIOLOGY

Role for early-differentiated natural killer cells in infectious mononucleosis

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Key Points

- Early-differentiated NK cells accumulate and proliferate during IM.
- These early-differentiated NK cells preferentially target lytic EBV-infected B cells in vitro.

A growing body of evidence suggests that the human natural killer (NK)-cell compartment is phenotypically and functionally heterogeneous and is composed of several differentiation stages. Moreover, NK-cell subsets have been shown to exhibit adaptive immune features during herpes virus infection in experimental mice and to expand preferentially during viral infections in humans. However, both phenotype and role of NK cells during acute symptomatic Epstein-Barr virus (EBV) infection, termed infectious mononucleosis (IM), remain unclear. Here, we longitudinally assessed the kinetics, the differentiation, and the proliferation of subsets of NK cells in pediatric IM patients. Our results indicate that acute IM is characterized by the preferential proliferation of early-differentiated CD56^{dim} NKG2A^{+} immunoglobulin-like receptor^{-} NK cells. Moreover, this NK-cell subset exhibits features of terminal differentiation and persists at higher frequency during at least the first 6 months after acute IM. Finally, we demonstrate that this NK-cell subset preferentially degranulates and proliferates on exposure to EBV-infected B cells expressing lytic antigens. Thus, early-differentiated NK cells might play a key role in the immune control of primary infection with this persistent tumor-associated virus. (*Blood*. 2014;124(16):2533-2543)

Introduction

Natural killer (NK) cells are a subset of innate lymphocytes that exhibit nonredundant antiviral functions in experimental mice.[1] In mice infected with the murine cytomegalovirus (MCMV), a subset of NK cells bearing the activating receptor Ly49H expands and persists at increased frequency for more than 2 months following primary infection. Notably, these cells display an enhanced protective response against MCMV in adoptive transfer experiments.[2] In humans, the peripheral blood compartment of NK cells is heterogeneous and accounts for 5% to 15% of lymphocytes. It is composed of diverse differentiation stages, which can be defined by the expression of surface markers, such as the 2 types of inhibitory receptors NKG2A and killer-cell immunoglobulin-like receptors (KIRs).[3,4] Human NK cells seem to play an important antiviral role, because patients with isolated NK-cell deficiencies exhibit an increased susceptibility to herpes viruses.[5] Furthermore, patients with acute viral infections resulting from hantavirus, cytomegalovirus (CMV), or chikungunya virus[6-8] accumulate the late-differentiated CD56^{dim} NKG2C^{+} KIR^{+} NK-cell subset in peripheral blood. However, none of these previous studies demonstrated a protective role for specifically accumulated human NK-cell subsets against virus-infected cells in vitro or in vivo.[9,10]

A ubiquitous persistent human virus, which has not been investigated in detail in this respect, is the primarily B-cell-tropic Epstein-Barr virus (EBV). EBV is a γ-herpes virus, which latently infects the vast majority of the adult human population worldwide, and is associated with B-cell and epithelial-cell malignancies.[11] EBV displays 2 modes of infection. One mode expresses latency genes (latent EBV) leading to B-cell transformation in vitro and subsequent generation of lymphoblastoid cell lines (LCLs). The other mode expresses lytic genes (lytic EBV) leading to the production of infectious viral particles and lysis of the host cell.[12] Most primary EBV infections occur before the age of 5 years and are usually asymptomatic. Nevertheless, primary EBV infection occurring beyond this age may manifest as infectious mononucleosis (IM) that affects around 10% of the population in Europe and the United States.[13,14] The usually self-limiting IM is characterized by a vigorous CD8^{+} T-cell response that mainly targets EBV lytic epitopes[15] and is associated with an increased risk of developing EBV-positive classic Hodgkin lymphoma.[16]

The contribution of particular NK-cell subsets to the immune control of EBV, especially during primary infection, remains elusive. Here, we examined how blood NK-cell subsets accumulate and

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respond during IM, and to what extent they can recognize latently and lytically EBV-infected B cells.

Material and methods

Study design

Twenty-two pediatric patients diagnosed with acute IM at the University Children's Hospital of Zurich were prospectively enrolled between October 2010 and April 2013. The onset date of symptoms was used as reference for the longitudinal study. Twelve pediatric patients with IM symptoms, but lacking the serological pattern compatible with acute EBV infection, were also enrolled (IM-like) and donated peripheral blood at diagnosis. All serum samples from IM-like patients were negative for HCMV DNA. Healthy children and healthy adults aged 20 to 30 years were used as healthy controls according to their EBV serology. Further details are outlined in the supplemental Methods available on the *Blood* Web site.

All participants provided informed consent in accordance with the Declaration of Helsinki, and the institutional ethics committee approved all protocols used.

Monoclonal antibodies and flow cytometry

Samples were acquired on a FACSCanto II and an LSR Fortessa (BD Biosciences). Details about the handling of PBMCs, flow cytometry analysis, and antibodies used are described in the supplemental Methods.

Cell lines

Preparation of viral stocks, cell lines used, and induction and isolation of lytic AKBM cells as well as the degranulation assay are described in the supplemental Methods.

Viral loads quantification

EBV DNA levels were determined by real-time polymerase chain reaction. The details of viral load measurements are outlined in the supplemental Methods.

Statistical analysis

Data were analyzed using Prism software (GraphPad Software, Inc.). *P* values of $<.05$ were considered significant and were calculated with the nonparametric Mann-Whitney *U* test or the Wilcoxon matched-pairs signed ranks tests. Spearman's rank correlation was used to examine associations between 2 quantitative values.

Results

Pediatric acute IM patients exhibit accumulation of activated $CD8^+$ T cells and $CD56^{dim}$ NK cells

In young adults, IM is characterized by vigorous T-cell responses mediated mainly by EBV-specific $CD8^+$ T cells.[15] Nevertheless, neither T-cell nor NK-cell responses in pediatric IM have been characterized. Because we examined pediatric IM patients only, we first assessed the dynamics of the T-cell and NK-cell subset responses in pediatric patients during the first 6 months of IM. Uninfected healthy individuals and pediatric patients with IM-like diseases were used as controls. Acute IM patients exhibited a twofold increased median frequency of $CD8^+$ T cells (Figure 1A), a 15-fold increased median frequency of activated $CD8^+$ T cells (Figure 1B), and 60-fold increased median HLA-DR^+ $CD8^+$ T-cell counts (data not shown) compared with controls. The numbers of activated T cells normalized within 6 months. These changes paralleled those of the cellular EBV DNA levels through time (Figure 1C). Neither EBV DNA levels nor frequency of $CD8^+$ T cells correlated with age (supplemental Figure 1). Therefore, pediatric IM patients as young as 2 years of age seem to exhibit the classic immunologic features found in young adults with IM.

Moreover, we observed a 1.7-fold increase in the median numbers of NK cells in acute IM compared with controls. These numbers returned to baseline levels after 1 month (Figure 1D). The blood NK-cell compartment is mainly composed of 2 well-characterized functional subsets, the $CD56^{bright}$ $CD16^-$ and the $CD56^{dim}$ $CD16^+$ subsets.[17] The former NK-cell subset produces large amounts of cytokines on monokine stimulation, acquires cytotoxicity only after prolonged activation, and is enriched in secondary lymphoid organs.[18] The latter NK-cell subset readily kills susceptible targets and can rapidly secrete IFN-γ on engagement of activating receptors.[19] Acute IM patients displayed unchanged counts of $CD56^{bright}$ $CD16^-$ NK cells (Figure 1F) and a 1.2-fold increase in the median count of $CD56^{dim}$ $CD16^+$ NK cells (Figure 1H) compared with controls. Interestingly, the intermediate NK-cell subset $CD56^{dim}CD16^-$ was increased in frequency during acute IM (Figure 1E and supplemental Figure 2A) and exhibited a 3.7-fold increase in median cell counts compared with controls (Figure 1G). Thus we found a selective increase of the total $CD56^{dim}$ NK-cell subset during acute IM.

Early-differentiated $CD56^{dim}$ $NKG2A^+$ KIR^- NK cells accumulate during IM and terminally differentiate as well as persist afterward

To dissect the $CD56^{dim}$ NK-cell subset, we analyzed the expression patterns of the inhibitory receptors NKG2A and KIRs, which might allow assessment of subtle maturation stages spanning from early-differentiated $CD56^{dim}$ $NKG2A^+$ KIR^- to late-differentiated $CD56^{dim}$ $NKG2A^-$ KIR^+ NK cells.[3,4] Acute IM patients exhibited a 1.8-fold increased median frequency of $CD56^{dim}$ $NKG2A^+$ KIR^- NK cells (Figure 2A-B), but a 1.5-fold reduced median frequency of $CD56^{dim}$ $NKG2A^-$ KIR^+ NK cells compared with EBV-negative and EBV-positive control individuals (Figure 2C). Moreover, acute IM patients displayed 4.8-fold and 3.5-fold increased median absolute numbers of $CD56^{dim}$ $NKG2A^+$ KIR^- NK cells compared with EBV-negative and EBV-positive control individuals, respectively (supplemental Figure 2B), but unchanged cell counts of the late-differentiated $CD56^{dim}$ $NKG2A^-$ KIR^+ NK cells. We observed no major changes in the frequencies and cell counts of the $CD56^{dim}$ $NKG2A^-$ KIR^- and $CD56^{dim}$ $NKG2A^+$ KIR^+ NK-cell subsets (supplemental Figure 2B-C). Furthermore, IM-like patients, that is, patients with IM symptoms but no acute EBV infection, did not exhibit NK-cell subset accumulation similar to that of IM patients. We did not find any differences in the frequency of this NK-cell subset between EBV-seronegative and EBV-seropositive control individuals. Surprisingly, the frequency of $CD56^{dim}$ $NKG2A^+$ KIR^- NK cells remained significantly elevated in longitudinally followed IM patients up to 6 months after acute IM, but returned to baseline after 2 years (Figure 2B).

CD57 has been proposed as a marker of terminal differentiation of NK cells[3,20] and has been shown to be upregulated on $CD56^{dim}$ $NKG2C^+$ NK cells during acute infection with CMV, hantavirus, or chikungunya virus.[6-8] Therefore, we hypothesized that if the accumulated $CD56^{dim}$ $NKG2A^+$ KIR^- subset found in acute IM patients is preferentially involved in the immune response against EBV, it should acquire CD57 during the acute phase of IM to

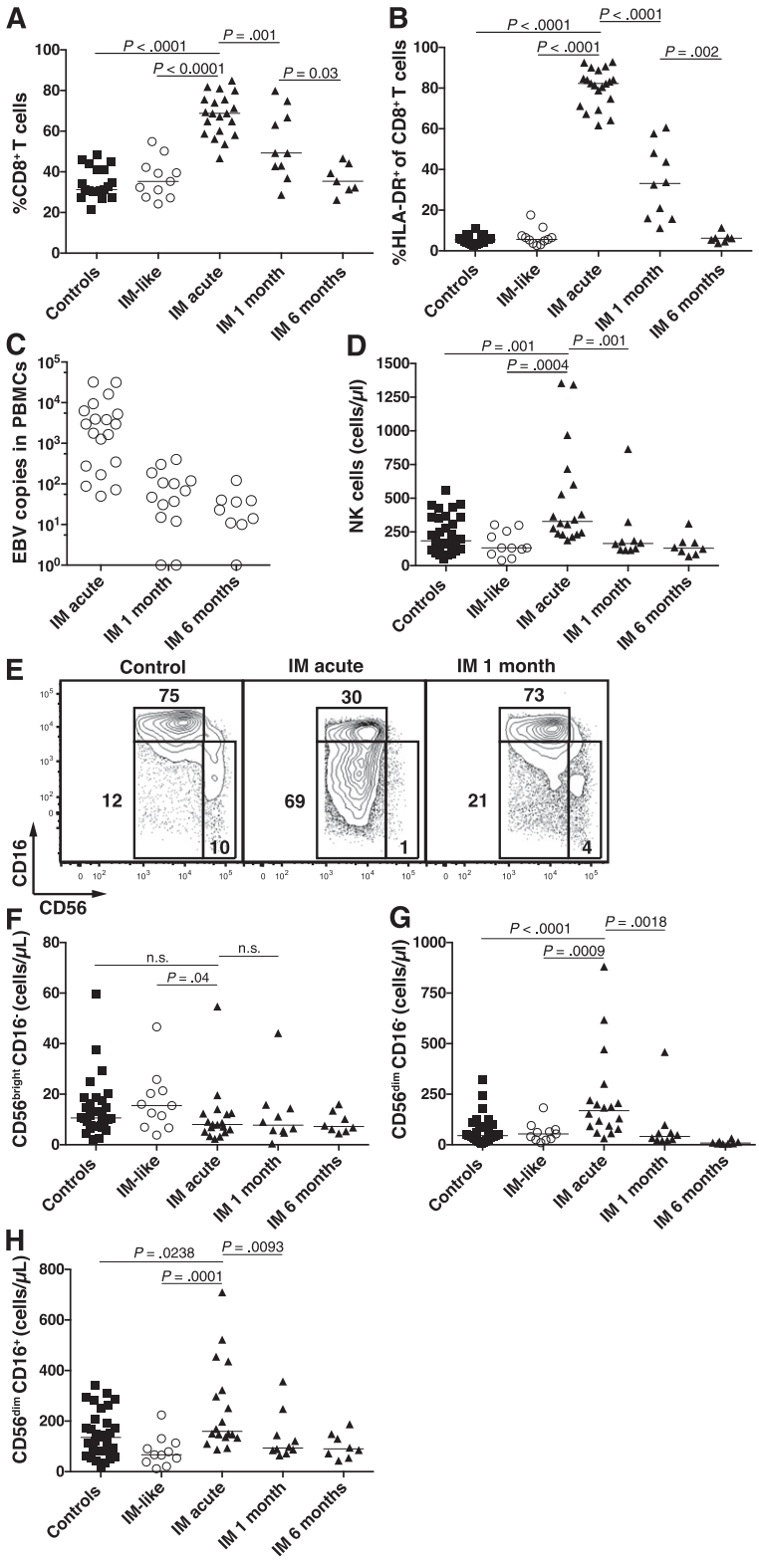

Figure 1. Accumulation of activated HLA-DR$^+$ CD8$^+$ T cells and CD56^{dim} NK cells during acute IM. PBMCs from healthy controls, IM-like patients, and IM patients at acute phase (IM acute), at 1 (IM 1 month), and 6 months (IM 6 months) were analyzed by flow cytometry. Frequencies of (A) CD8$^+$ T cells within the CD3$^+$ T-cell population and (B) HLA-DR$^+$ CD8$^+$ T cells within the CD8$^+$ T-cell population in healthy controls (n = 19), IM-like (n = 11), and IM acute (n = 20), 1-month (n = 10) and 6-month (n = 7) patients. (C) EBV DNA load in copies per 10^6 PBMCs in IM-acute (n = 19), 1-month (n = 14), and 6-month (n = 9) patients. Counts (cells/μL blood) of total NK cells (D) and frequencies of CD56^{bright} CD16$^-$, CD56^{dim} CD16$^-$, and CD56^{dim} CD16$^+$ NK-cell subsets within the CD3$^-$ CD56$^+$ NK-cell population from representative healthy control, IM-acute and 1-month patient (E). Counts (cells/μL blood) of (F) CD56^{bright} CD16$^-$, (G) CD56^{dim} CD16$^-$, and (H) CD56^{dim} CD16$^+$ NK cells in healthy controls (n = 31), IM-like patients (n = 11), and IM-acute (n = 18), 1-month (n = 10), and 6-month (n = 8) patients.

complete its terminal differentiation. Indeed, we observed a 2.5-fold increase in the median frequency of CD57$^+$ within the CD56^{dim} NKG2A$^+$ KIR$^-$ subset from the acute IM phase to 1 month later, but no changes in the CD56^{dim} NKG2A$^-$ KIR$^+$ subset (Figure 2D-E, respectively). Thus, acute symptomatic EBV infection elicits the specific accumulation of the CD56^{dim} NKG2A$^+$ KIR$^-$ NK-cell subset, its terminal differentiation, as well as its persistence at higher frequency during the first 6 months after acute IM.

IM patients exhibit a stable KIR repertoire and unchanged frequencies of CD56^{dim} NKG2C$^+$ NK cells

The KIR repertoire is composed of several activating and inhibitory receptors specific for distinct groups of HLA class I alleles,[21] is

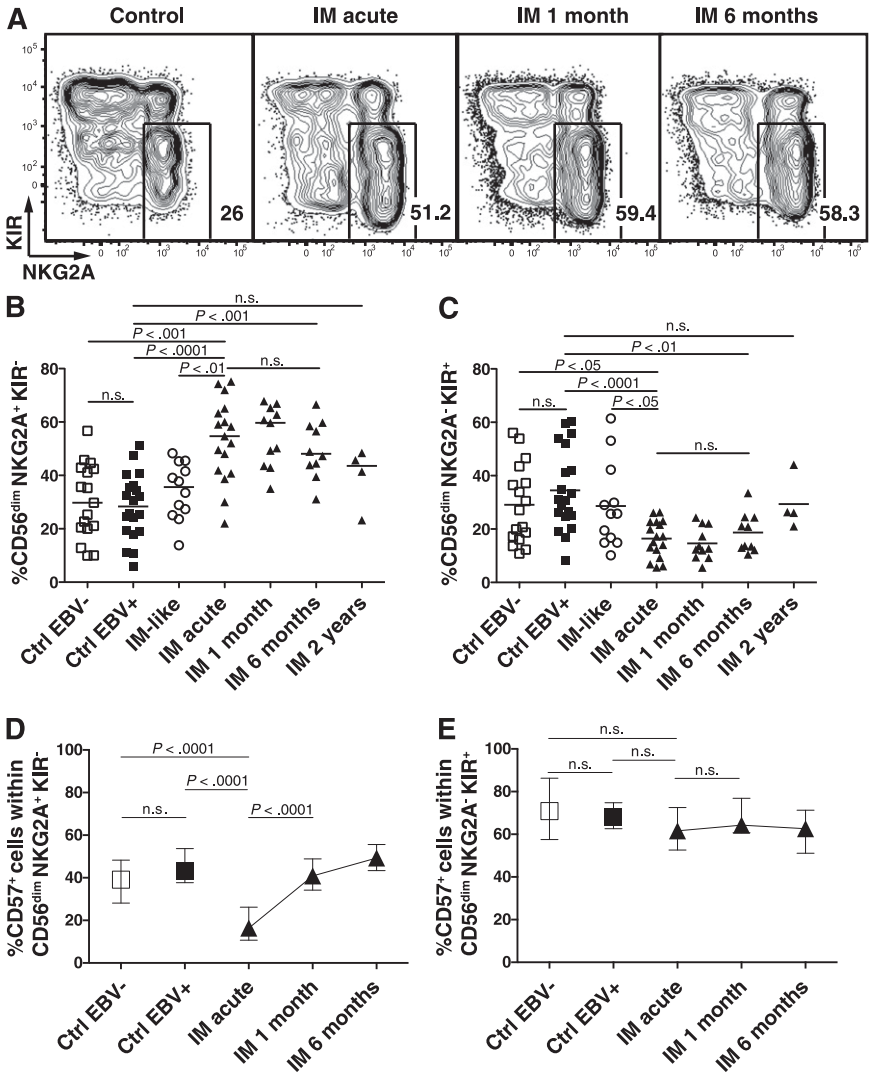

Figure 2. Accumulation and terminal differentiation of the CD56^{dim} NKG2A^{+} KIR^{-} NK-cell subset during acute IM. PBMCs from controls and IM patients were analyzed by flow cytometry. (A) Frequencies of CD56^{dim} NKG2A^{+} KIR^{-} NK cells within the CD56^{dim} population from representative healthy control, IM-acute, 1-month, and 6-month patients. Frequencies of (B) CD56^{dim} NKG2A^{+} KIR^{-} and (C) CD56^{dim} NKG2A^{-} KIR^{+} NK cells in healthy EBV-negative controls (n = 17), healthy EBV-positive controls (n = 20), IM-like patients (n = 12), and IM-acute (n = 17), 1-month (n = 11), 6-month (n = 10), and 2-year (n = 4) patients. Frequencies of CD57^{+} cells within (D) the CD56^{dim} NKG2A^{+} KIR^{-} and (E) the CD56^{dim} NKG2A^{-} KIR^{+} NK-cell subsets in healthy EBV-negative controls (n = 17), healthy EBV-positive controls (n = 20), and IM-acute (n = 17), 1-month (n = 11), 6-month (n = 10), and 2-year (n = 4) patients. Horizontal lines or single symbols indicate median values. Error bars indicate interquartile ranges. Mann-Whitney *U* tests.

highly variable among individuals, and is stable throughout time in healthy adults.[22] Particular KIR receptors might be involved in the immune control of viruses such as HIV,[23-26] the hepatitis C virus,[27,28] or CMV.[22,29] Therefore, to ensure that IM does not lead to an accumulation of NK cells bearing specific KIRs, we performed a comprehensive phenotypic KIR analysis[30] in a group of CMV-seronegative healthy controls and IM patients. The KIR repertoire of IM patients remained stable throughout the first month of IM (Figure 3A), and the frequencies of single KIR^{+} CD56^{dim} NK cells in IM patients were overall low compared with controls (Figure 3B). Thus, IM is associated with the accumulation of a CD56^{dim} NKG2A^{+} NK-cell subset, which does not carry any increase in activating, nor inhibitory, KIR molecule expression. Furthermore, CD56^{dim} NK cells expressing NKG2C, the activating counterpart of NKG2A,[31] accumulate on CMV infection,[7,32,33] as well as on other viral infections in CMV-positive individuals.[6,8,34-36] Thus, we assessed whether a similar accumulation occurs on EBV infection and therefore investigated CMV-seronegative IM patients and controls to avoid bias associated with CMV carriage.[37] We observed no changes in the frequency of NKG2C^{+} NK cells within the CD56^{dim} NKG2A^{-} NK-cell subset (Figure 3C-D) nor within the expanding CD56^{dim} NKG2A^{+} KIR^{-} NK-cell subset (data not shown). Thus, CD56^{dim} NKG2A^{+} KIR^{-} NKG2C^{-} NK cells accumulate during IM.

The preferential proliferation of CD56^{dim} NKG2A^{+} KIR^{-} NK cells positively correlates with cellular EBV loads during acute IM

We next investigated whether the increase in the absolute numbers of CD56^{dim} NKG2A^{+} KIR^{-} NK cells might be caused by active proliferation of this NK-cell subset. We assessed the expression of the proliferation marker Ki-67 in the CD56^{bright}, the CD56^{dim} NKG2A^{+} KIR^{-}, and the CD56^{dim} NKG2A^{-} KIR^{+} NK-cell subsets in acute IM 1 month later. We found a twofold and a threefold increase in the median frequency of Ki-67^{+} cells within the CD56^{dim} NKG2A^{+} KIR^{-} NK cells in acute IM compared with controls and IM at 1 month, respectively (Figure 4A-B). However, we did not observe any increased proliferation in the more differentiated NKG2A^{-} KIR^{+} NK subset, and there was no difference when comparing EBV-negative with EBV-positive controls (Figure 4A-B and data not shown). In addition, the precursor CD56^{bright} NK-cell subset, which exhibits strong proliferation properties and responds to minute doses of cytokines in vitro, did not show an increased frequency of Ki-67^{+} cells in acute IM patients (Figure 4B). Notably, the frequencies of proliferating Ki-67^{+} cells within the CD56^{dim} NKG2A^{+} KIR^{-} positively correlated with EBV DNA levels in PBMCs (Figure 4C), but not in serum (data not shown). No such correlation was observed within the CD56^{dim} NKG2A^{-} KIR^{+} NK-cell subset. This suggested that EBV-infected B cells in IM patients might directly drive the proliferation of early-differentiated CD56dim NK cells. We next asked

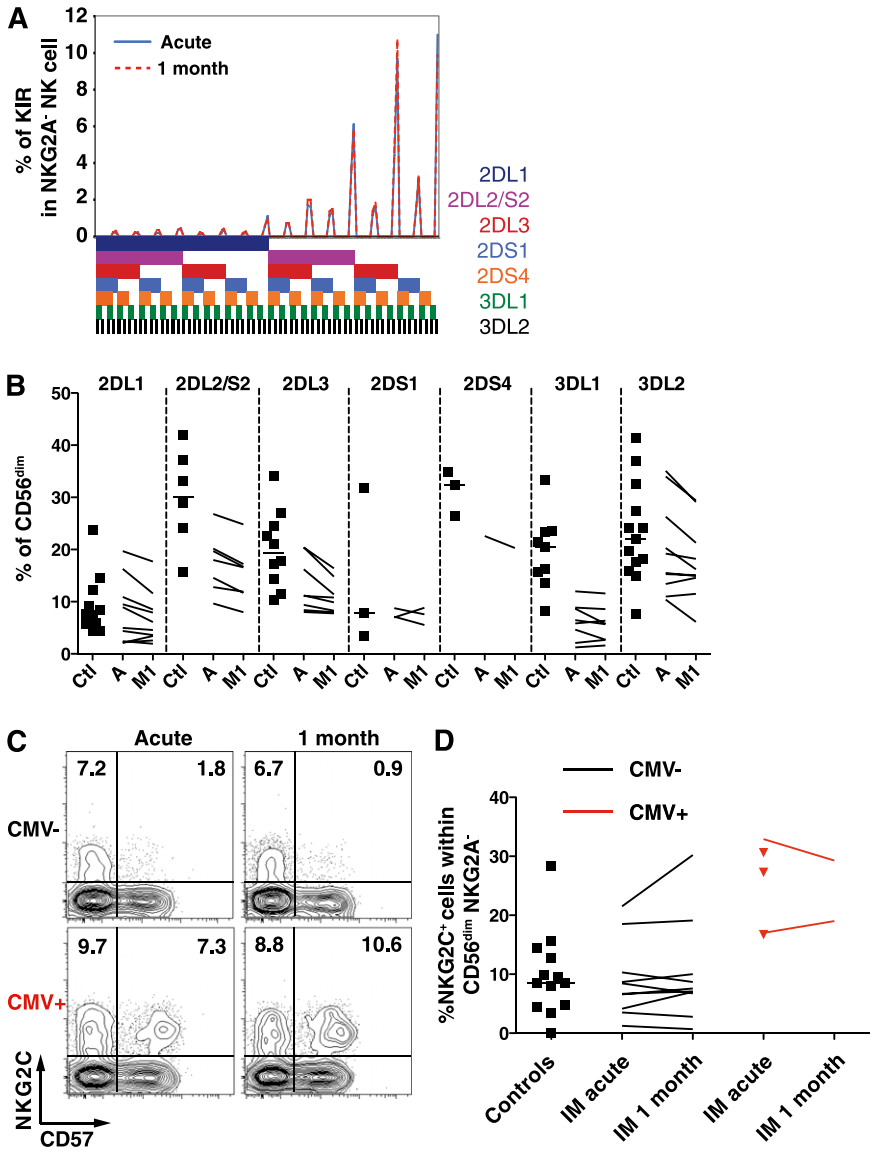

Figure 3. Frequencies of single KIR-positive and NKG2C$^+$ CD56^{dim} NK cells are not altered during acute IM. (A) Frequencies of CD56^{dim} NKG2A$^-$ NK cells expressing the 7 analyzed KIRs from 1 representative CMV-seronegative IM patient at acute phase and at 1 month. The presence of 1 KIR in a combination is represented by a color code below the graph. (B) Frequencies of single KIR-positive CD56^{dim} NK cells in healthy controls (Ctl, n = 11) and IM patients (n = 10) at acute phase (A) and at 1 month (M1). (C) Frequencies of NKG2C$^+$ CD57$^+$ NK cells within the CD56^{dim} NKG2A$^-$ population from 1 CMV-seronegative and 1 CMV-seropositive IM patient at acute phase and at 1 month. (D) Frequencies of NKG2C$^+$ NK cells within the CD56^{dim} NKG2A$^-$ NK cell population in CMV-seronegative healthy controls (n = 13), CMV-seronegative (n = 10), and CMV-seropositive (n = 5, red) IM patients at acute phase and at 1 month (n = 2 for CMV-seropositive, red lines).

whether the proliferation of the CD56^{dim} NKG2A$^+$ KIR$^-$ NK-cell subset differs according to CD57 expression. Surprisingly, proliferation was exclusively found within the CD57$^-$ fraction (Figure 4D-E). Our finding aligns with previous studies showing a decreased proliferative potential of CD57$^+$ NK cells compared with CD57$^-$ NK cells.[3,20] Thus, CD56^{dim} NKG2A$^+$ KIR$^-$ CD57$^-$ NK cells seem to proliferate preferentially during acute IM but not 1 month later. Moreover, in the acute phase, proliferation parallels the accumulation of this NK-cell subset, which displays a sevenfold and a 4.8-fold median increase compared with EBV-negative and EBV-positive controls, respectively (Figure 4F). In addition, CMV status did not seem to influence the NK-cell response (supplemental Figure 3). We did not observe any correlation between the count of the CD56^{dim} NKG2A$^+$ KIR$^-$ CD57$^-$ NK cells, nor the count of total NK cells, and the EBV DNA levels in PBMCs or in serum (data not shown). Thus, early-differentiated NK cells accumulate in IM patients after EBV-driven proliferation.

CD56^{dim} NKG2A$^+$ KIR$^-$ NK cells preferentially target EBV-infected B cells with lytic reactivation

CD56^{dim} NKG2A$^+$ KIR$^-$ NK cells are functional against HLA-class-I-deficient target cells, including the EBV-positive LCL 721.221 cell line,[4] but their reactivity toward HLA class I competent autologous LCLs has not yet been assessed. We observed a low overall frequency of degranulating NK cells on coculture with autologous LCLs. Nevertheless, the CD56^{dim} NKG2A$^+$ KIR$^-$ NK subset, which accumulates during IM, displayed a more than twofold increase in degranulation compared with the CD56^{bright} and CD56^{dim} NKG2A$^-$ KIR$^+$ NK-cell subset (Figure 5A). In contrast, the EBV-negative allogeneic B-cell line L428, which exhibits an activated phenotype comparable to LCLs, elicits an increased response in the CD56^{dim} NKG2A$^-$ KIR$^+$ NK subset only (Figure 5B). This low NK-cell response against autologous LCLs might be a result of their high surface level of HLA class I and HLA-E which might engage the NK-cell inhibitory receptors KIR and NKG2A, respectively. Indeed, EBV-infected B cells upregulated HLA class I[38] and HLA-E (Figure 5C).

On the other hand, induction of the lytic cycle of EBV infection has been shown to sensitize EBV-infected B cells to NK-cell killing using the EBV-positive Akata Burkitt lymphoma reporter cell line AKBM,[39] which allows the purification of Akata Burkitt lymphoma cells with and without EBV lytic reactivation, respectively. Hence, we tested the degranulation of the CD56^{dim} NKG2A$^+$ KIR$^-$ NK subset against either latent AKBM or lytic AKBM cells from convalescent

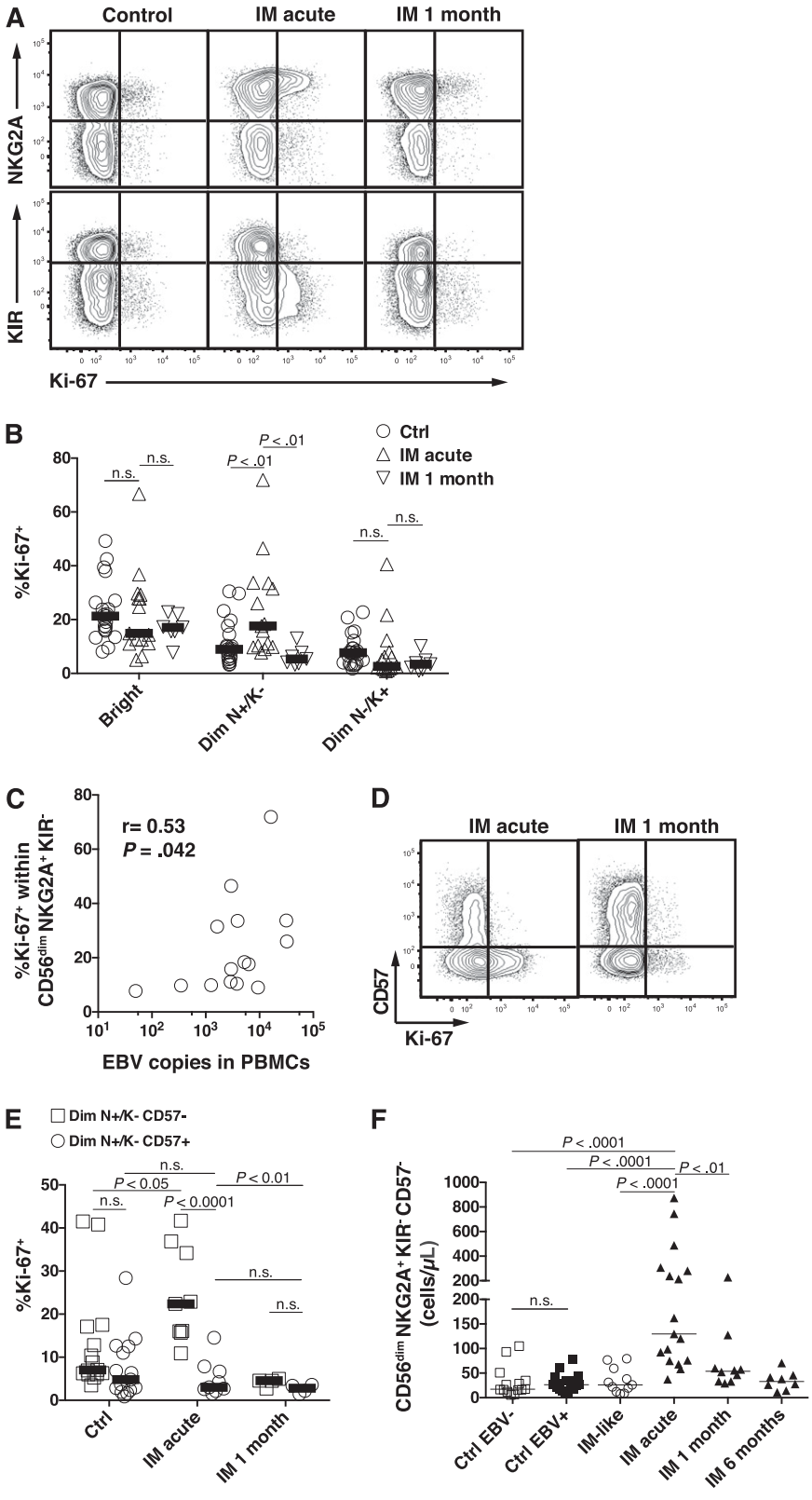

Figure 4. Increased count of CD56^{dim} NKG2A^{+} KIR^{-} CD57^{-} NK cells during acute IM is caused by preferential proliferation. (A) Representative examples of costaining for NKG2A and Ki-67 and costaining for KIR and Ki-67 on CD56^{dim} NK cells in healthy control and IM-acute and 1-month patients. (B) Frequencies of Ki-67^{+} cells within the CD56^{bright}, CD56^{dim} NKG2A^{+} KIR^{-}, and CD56^{dim} NKG2A^{-} KIR+ NK-cell subsets in healthy controls (n = 21), in IM-acute (n = 15), and in 1-month patients (n = 7). (C) Correlation of EBV DNA loads (copies per 10^{6} PBMCs) and frequencies of Ki-67^{+} cells within the CD56^{dim} NKG2A^{+} KIR^{-} NK-cell subset from acute IM patients. Spearman r = 0.53, *P* (2-tailed) = .042. (D) Representative example of costaining for CD57 and Ki-67 on CD56^{dim} NKG2A^{+} KIR^{-} NK cells in an IM-acute and 1-month patient. (E) Frequencies of Ki-67^{+} cells within the CD56^{dim} NKG2A^{+} KIR^{-} NK cells according to CD57 expression in healthy controls (n = 15), IM-acute (n = 9), and 1-month (n = 4) patients. (F) Count of CD56^{dim} NKG2A^{+} KIR^{-} CD57^{-} in healthy EBV-negative controls (n = 14), healthy EBV-positive controls (n = 17), IM-like (n = 11), IM-acute (n = 17), 1-month (n = 10), and 6-month (n = 8) patients. Horizontal lines indicate median values of a given symbol. Mann-Whitney *U* tests.

IM patients and healthy EBV-positive controls and compared it to the CD56^{bright} and the CD56^{dim} NKG2A^{-} KIR^{+} subsets. To avoid HLA class I/KIR mismatch bias, we specifically assessed the degranulation in KIR^{+} matched (KIR2DL2/DL3/3DL1^{+}) NK cells according to the AKBM genotype (Bw4/C1/C1). We could confirm increased responses of NK cells against lytic compared with latent AKBM cells (Figure 5D-F) previously shown to be mediated by a down-regulation of the inhibitory ligands HLA-class I and HLA-E and an upregulation of the activating ligands CD112 and ULBP-1.[39] Similarly, we also found increased expression of activating ligands

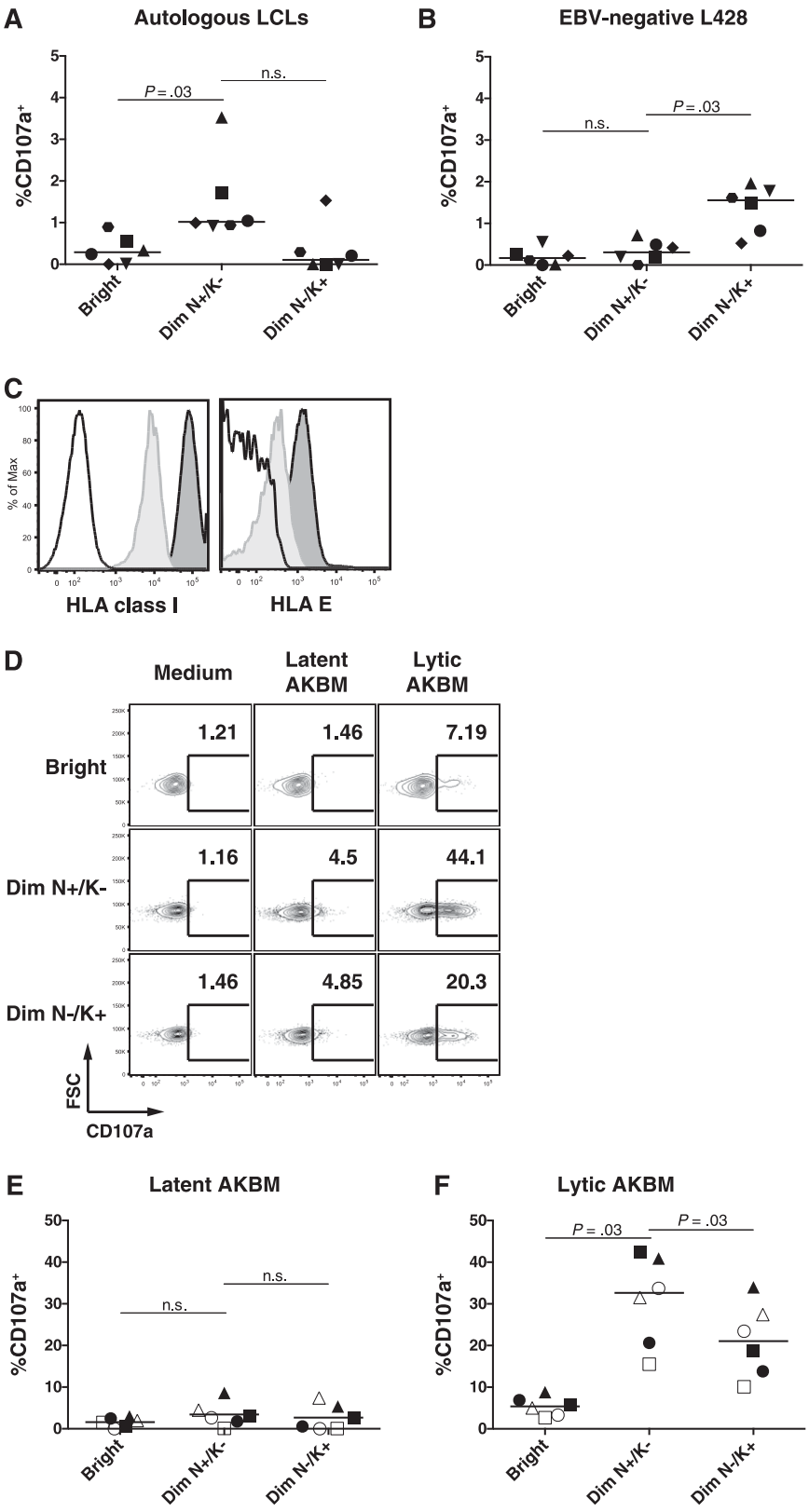

Figure 5. Increased cytotoxic degranulation of the CD56^{dim} NKG2A^{+} KIR^{-} NK subset against EBV-infected B cells with lytic replication. (A) PBMCs from 6 healthy EBV-positive donors were cocultured with autologous LCLs and (B) EBV-negative L428 at an effector to target ratio of 10:1 for 6 hours. Frequencies of degranulating (CD107a^{+}) cells within the CD56^{bright} (Bright), the CD56^{dim} NKG2A^{+} KIR^{-} (Dim N+/K−), and the CD56^{dim} NKG2A^{-} KIR^{+} (Dim *N*−/K+) NK-cell subsets were assessed by flow cytometry at the end of the coculture. (C) HLA class I and HLA-E expression on CD19^{+} B cells from PBMCs (light gray histogram) and from autologous LCLs (dark gray histogram). Isotype controls are depicted as a white histogram. (D) Representative example of frequencies of CD107a^{+} NK cells within the 3 NK-cell subsets after coculture with latent AKBM or lytic AKBM. Frequencies of degranulating (CD107a^{+}) NK cells within the CD56^{bright} (Bright), the CD56^{dim} NKG2A^{+} KIR^{-} (Dim N+/K−) and the CD56^{dim} NKG2A^{-} matched KIR^{+} (Dim *N*−/K+) NK-cell subsets in PBMCs from 3 convalescent IM patients (open symbols) and 3 healthy EBV-positive donors (filled symbols) cocultured with (E) latent AKBM or (F) lytic AKBM (n = 6). Horizontal lines indicate median values of a given subset, Wilcoxon matched-pairs signed ranks tests.

on lytic EBV infected LCLs (supplemental Figure 4D-G), however the expression of the respective activating receptors in the CD56^{dim} NKG2A^{+} KIR^{-} NK-cell subset was unaltered in IM patients (supplemental Figure 4A-C). Importantly, the CD56^{dim} NKG2A^{+} KIR^{-} NK-cell subset exhibited a significantly stronger degranulation against lytic AKBM cells compared with all other subsets (Figure 5F). We found no difference in the degranulation capacity of CD56^{dim} NKG2A^{+} KIR^{-} NK between convalescent IM patients and controls, suggesting that these NK cells are functional and are not in an exhausted state in the aftermath of acute IM. Thus,

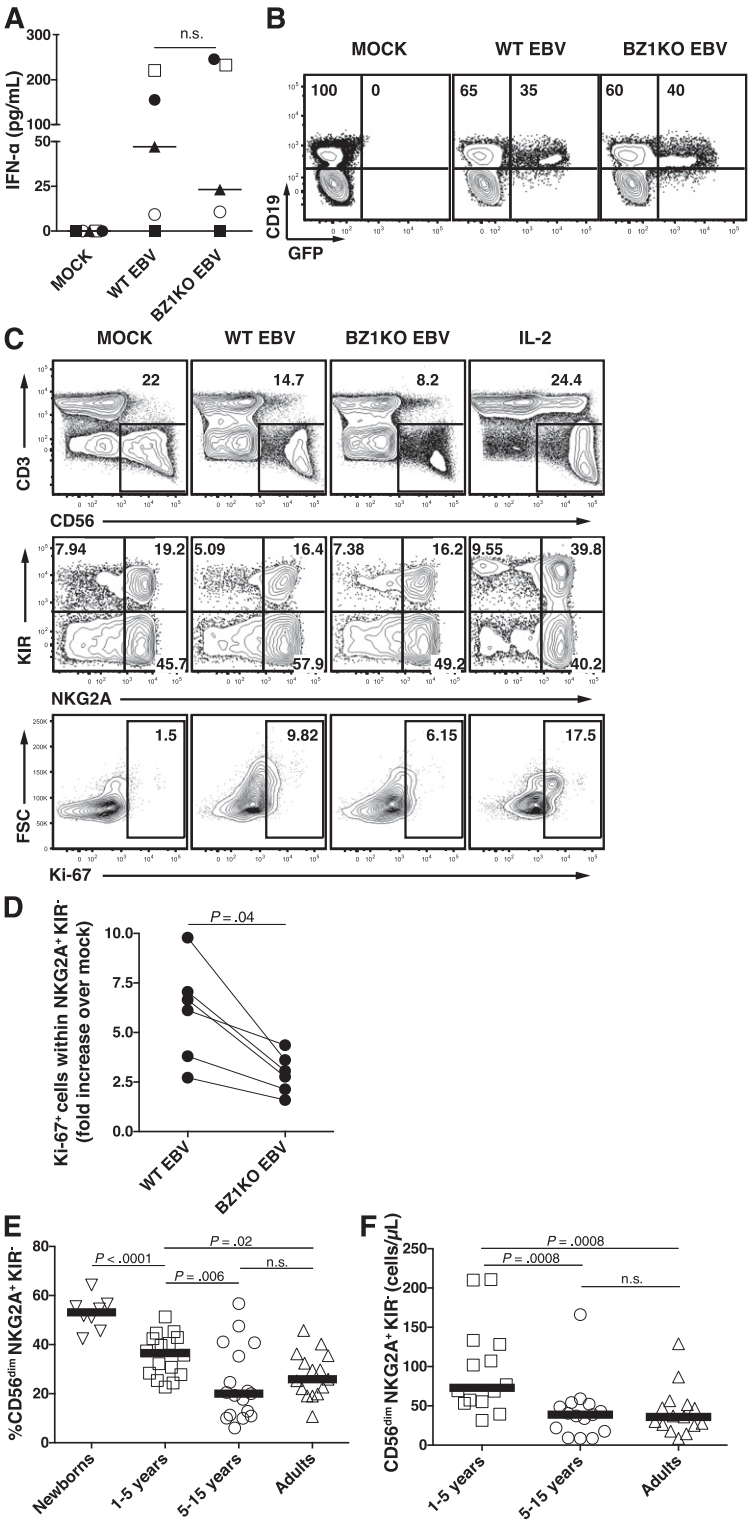

Figure 6. EBV-driven in vitro proliferation of NKG2A$^+$ KIR$^-$ NK cells partially depends on expression of lytic antigens. Proliferation of NK-cell subsets was assessed 7 days after inoculation of CBMCs with either WT EBV, BZLF1-KO (BZ1KO) EBV or PBS (MOCK). (A) Concentrations of IFN-α in supernatant 24-hour postinoculation on MOCK, WT EBV, and BZ1KO EBV infection (pg/mL; n = 5). (B) Representative example of CD19 and GFP costaining within live lymphocytes 72 hours postinfection. Numbers indicate frequencies of GFP-negative or GFP-positive cells within the CD19$^+$ B-cell population. (C) Frequencies of Ki-67$^+$ NKG2A$^+$ KIR$^-$ NK cells 7 days after inoculation of CBMCs with mock, WT EBV, or BZ1KO EBV, or after stimulation with IL-2. The depicted gates were assessed within live lymphocytes (first row), CD3$^-$ CD56$^+$ NK cells (second row), and NKG2A$^+$ KIR$^-$ NK cells (third row). (D) Ratio of NKG2A$^+$ KIR$^-$ Ki-67$^+$ NK-cell counts from WT EBV- or BZ1KO EBV- over mock-infected CBMCs. (E) Frequencies of CD56^{dim} NKG2A$^+$ KIR$^-$ NK cells in newborns (n = 8), children aged 1 to 5 years (n = 16), children aged 5 to 15 years (n = 17), and adults aged 20 to 30 years (n = 15). (F) Counts of CD56^{dim} NKG2A$^+$ KIR$^-$ NK cells in children aged 1 to 5 years (n = 14), children aged 5 to 15 years (n = 15), and adults aged 20 to 30 years (n = 15). Horizontal lines indicate median values of a given age group, Mann-Whitney *U* tests.

CD56^{dim} NKG2A$^+$ KIR$^-$ NK cells preferentially recognize lytic EBV-replicating B cells.

EBV lytic replication triggers in vitro proliferation of NKG2A$^+$ KIR$^-$ NK cells

Finally, we examined the proliferation of NKG2A$^+$ KIR$^-$ NK cells using staining for Ki-67[40] in an in vitro model of primary EBV infection of cord blood mononuclear cells (CBMC) infected with either wild-type (WT) EBV or lytic replication incompetent BZLF1-KO (BZ1KO) EBV. Both viruses elicited similar IFN-type I responses 24 hours postinfection (Figure 6A) and exhibited comparable infection capacity, as evaluated by the frequencies of GFP$^+$ EBV-infected B cells 3 days postinfection (Figure 6B). Because most NK cells upregulated CD56 surface expression during in vitro culture (Figure 6C, first row), we did not distinguish between the CD56^{bright}

and CD56^{dim} NK cells for further analysis. We observed an increased proliferation of NKG2A$^+$ KIR$^-$ NK cells 7 days after infection with WT EBV compared with mock (Figure 6C-D). Infection with BZ1KO EBV, with abolished expression of all EBV lytic antigens, elicited a twofold-reduced proliferation of NKG2A$^+$ KIR$^-$ NK cells, in comparison with WT EBV (median ratio 2.9 vs 6.3, $P = .04$). This indicates that the proliferation of NKG2A$^+$ KIR$^-$ NK cells partially depends on the presence of EBV-infected cells expressing lytic antigens. Notably, NKG2A$^-$ KIR$^+$ NK cells exhibit similar proliferation on infection with WT EBV, but this is not affected by the absence of lytic antigens (data not shown). Finally, we assessed a possible age-dependent distribution of this early-differentiated NK-cell subset in peripheral blood from healthy individuals that might correlate with the known age-dependent prevalence of primary symptomatic EBV infection. Indeed, CD56^{dim} NKG2A$^+$ KIR$^-$ NK cells both decreased in frequency (Figure 6E) and in absolute numbers (Figure 6F) during the first decade of life, whereas the counts of CD56^{dim} NKG2A$^-$ KIR$^+$ NK cells remained unchanged with age (data not shown). Thus, CD56^{dim} NKG2A$^+$ KIR$^-$ NK cells, which decrease in frequency in the first decade of life, preferentially degranulate and proliferate in response to lytic EBV-replicating B cells.

Discussion

Here we demonstrate in longitudinally followed pediatric IM patients that an early-differentiated CD56^{dim} NKG2A$^+$ KIR$^-$ NK subset selectively accumulates during primary symptomatic EBV infection and persists at increased frequencies for months. Moreover, our data indicate that these NK cells specifically recognize B cells undergoing lytic EBV replication. Our findings are unprecedented and suggest that responses of NK-cell subsets to viral infections may not be confined to late-differentiated populations.[6-8] Moreover, distinct NK-cell subsets may be rather pathogen-specific.

Remarkably, although we found increased counts in the cytotoxic CD56^{dim} NK-cell subset, but not in the less-differentiated CD56^{bright} CD16$^-$ NK-cell subset, we could not confirm the previously reported expansion of CD56^{bright} CD16$^+$ NK cells during acute symptomatic EBV infection.[41] We rather observed an unusual increase of the intermediate CD56^{dim} CD16$^-$ NK-cell subset, which might be explained by downregulation of CD56 on CD56^{bright} NK cells or by downregulation of CD16 such as observed in degranulating CD56^{dim} NK cells on coculture with K562 cells (unpublished observations). This CD56^{dim} NK-cell subset is distinctly characterized by NKG2A expression and by the absence of KIRs. It strikingly differs from that of other acute viral infections such as CMV, hantavirus, or chikungunya virus infection,[6-8] in which the CD56^{dim} KIR$^+$ NKG2C$^+$ NK-cell subset was shown to be expanded. CD56^{dim} NKG2A$^+$ KIR$^-$ NK cells are considered early-differentiated[4] as suggested by the specific temporal reconstitution of the NK-cell subsets in hematopoietic stem-cell transplanted patients[42-44] and in mice with human immune-system components.[3] Based on our results, ongoing differentiation of these early-differentiated NK cells seems to occur during the first weeks of IM; this is further supported by studies in EBV-infected mice with human immune-system components.[45]

Another striking feature of the IM-associated NK-cell response is the persistence of elevated frequencies of the CD56^{dim} NKG2A$^+$ KIR$^-$ NK cells for up to 6 months after CD8$^+$ T-cell numbers have normalized. Nevertheless, we observed no difference in the peripheral blood frequencies of these NK cells between EBV-seropositive and EBV-seronegative control individuals, contrasting the situation after CMV infection where late differentiated NK cells persist at increased levels.[32] This might be explained by compartmentalized NK-cell accumulation during asymptomatic EBV infection, as has been proposed for EBV-specific T-cell responses in the tonsils,[47,48] and suggests that the acute symptomatic EBV infection systemically imprints the NK-cell compartment differently. Accordingly, CD56^{bright} NKG2A$^+$ and mostly KIR$^-$ NK cells were found enriched in tonsils of EBV-seropositive compared with EBV-seronegative individuals.[49] Indeed, this could result from ongoing lytic EBV replication at these sites in asymptomatic EBV carriers.[46] Additionally, we determined that the increased CD56^{dim} NKG2A$^+$ KIR$^-$ NK-cell numbers during acute IM were caused by the selective proliferation of this subset. Such selectivity has not been reported to our knowledge during other acute viral infections. Because the CD56^{dim} NKG2A$^+$ KIR$^-$ NK subset only actively proliferated during the acute phase of IM and was only increased in absolute numbers at this stage, the persistently increased frequency of this subset might be caused by either a longer survival of these NK cells in peripheral blood or a continuous EBV-driven proliferation in tissues followed by recruitment and accumulation in the peripheral blood. Indeed, the numbers of EBV-infected B cells quickly decrease in the peripheral blood after acute IM,[50] and EBV turns off antigen expression in these cells.[51] Thus, an EBV-driven proliferation of NK cells during IM convalescence might not be expected in peripheral blood. Nevertheless, IM patients exhibit prolonged oral EBV shedding up to 1 year after IM,[14,52] indicating that EBV replication occurs in the oropharynx. This, in turn, may result in local EBV-driven proliferation of NK cells after IM that are subsequently recruited in peripheral blood.

Several lines of evidence support the hypothesis that EBV-infected B cells, and not proinflammatory cytokines, directly drive the unique proliferation of early-differentiated NK cells: first, a similar expansion is not observed in patients with IM-like symptoms; second, the precursor CD56^{bright} NK-cell subset, which possesses strong proliferative responses to cytokines, does not display an enhanced proliferation during acute IM; third, the frequency of proliferating early-differentiated NK cells positively correlates with EBV loads in blood cells. On the other hand, we did not observe any correlation between overall NK-cell counts and viral load. These findings are in conflict with previous studies reporting a negative correlation between NK-cell frequencies and counts and cellular EBV DNA levels[41] or a positive correlation between NK-cell counts and EBV DNA levels in whole blood.[14] Hence, acute IM elicits the proliferation of CD57$^-$ CD56^{dim} NKG2A$^+$ KIR$^-$ NK cells, which then differentiate to become CD57 positive. A fraction of this subset might represent antigen-experienced NK cells that specifically responded to EBV-infected cells in a similar fashion as during MCMV infection of experimental mice in which these NK cells are suggested to constitute memory NK cells.[2,53]

These early-differentiated NK cells exhibit enhanced degranulation against EBV-infected B cells with lytic reactivation. Reduced HLA-E-mediated inhibitory signals on EBV-infected cells expressing lytic antigens might lead to preferential recognition by NKG2A$^+$ NK cells and could be caused by decreased availability of class I leader signal peptide[39] or via direct modulation of HLA-E by EBV-derived peptides.[54] In addition to these diminished inhibitory signals, we and others have observed an increased expression of activating ligands in lytic EBV infection,[39] and NKG2D and DNAM1 have been identified as the main activating NK-cell receptors for the recognition of B cells with lytic EBV infection in vitro.[39] In good agreement, loss of NKG2D function has been found to confer susceptibility to uncontrolled EBV infection and neoplasia in XMEN

patients in vivo.[55] How lytic EBV infection leads to the upregulation of activating ligands, however, remains unclear. NKG2D ligands have been reported to be upregulated on induction of the DNA damage response and cytosolic DNA recognition.[56] Although EBV, like other herpes viruses, packages its DNA into the viral capsid in the nucleus during lytic replication, some of it might become accessible during capsid transit through the cytosol and trigger this pathway of NKG2D ligand upregulation. Newly infected B cells transiently express several lytic antigens, mostly from the immediate early and early lytic genes,[57] and some of these antigens might contribute to the proliferation of NKG2A$^+$ KIR$^-$ NK cells on EBV infection of CBMCs.

We suggest that CD56^{dim} NKG2A$^+$ KIR$^-$ NK cells preferentially recognize autologous B cells with lytic EBV infection, and that recognition of lytic EBV replication drives the characteristic NK-cell accumulation that we observed in acute IM patients. Because IM only manifests in older children, adolescents, and young adults with decreased frequencies of this herein newly described EBV-reactive NK-cell subset, we postulate an age-dependent impaired NK-cell-mediated immune control of EBV infection as one possible cause of IM.

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Authorship

Contribution: T.A. designed and performed research; A.L., A.M., V.B., and K.-J.M. performed experiments and analyzed data; C.M., O.C., and D.N. designed research; S.U., G.S., C.G., and C.B. contributed essential information or vital reagents; T.A. and O.C. analyzed data; and T.A., C.M., O.C., and D.N. wrote the manuscript.

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MAJOR ARTICLE

Oropharyngeal Group A Streptococcal Colonization Disrupts Latent Epstein-Barr Virus Infection

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Epstein-Barr virus (EBV) infects >90% of the human population within the first 2 decades of life and establishes reversible latent infection in B cells. The stimuli that lead to switching from latent to lytic EBV infection in vivo are still elusive. Group A streptococci (GAS) are a common cause of bacterial pharyngotonsillitis in children and adolescents and colonize the tonsils and pharynx of up to 20% of healthy children. Thus, concomitant presence of EBV and GAS in the same individual is frequent. Here, we show that EBV carriers who are colonized with GAS shed EBV particles in higher numbers in their saliva, compared with EBV carriers not colonized with GAS. Messenger RNA levels of the master lytic regulatory EBV gene *BZLF1* were more frequently detected in tonsils from EBV carriers colonized with GAS than from EBV carriers not colonized. Heat-killed GAS, potentially mimicking GAS colonization, elicited lytic EBV in latently infected lymphoblastoid cell lines (LCLs) partially via Toll-like receptor 2 triggering, as did purified GAS peptidoglycan. Thus, colonization by GAS might benefit EBV by increasing the EBV load in saliva and thereby enhancing the likelihood of EBV spread to other hosts.

Keywords. Epstein-Barr virus (EBV); group A *Streptococci* (GAS); tonsil; oropharynx; lytic; latent; TLR2; salivary shedding.

More than 90% of the adult population is persistently and asymptomatically infected with Epstein-Barr virus (EBV), a human B-lymphotropic γ-herpesvirus [1]. Primary infection with EBV is acquired mainly in childhood via saliva [1]. The oropharynx is the portal of entry and exit, where the palatine tonsils act as reservoir for EBV [2–4]. In its latent form in B cells, EBV expresses a limited number of genes, does not replicate, and its DNA is propagated to daughter cells during cell division. By contrast, in its lytic form, EBV expresses genes required for replication and generation of infectious virus particles resulting in host cell lysis and virus release [5]. The default state of EBV infection is the latent form, which is reversible, permitting the creation of new viral particles and transmission to other hosts [6]. The mechanisms responsible for switching to the lytic form in vivo are not completely understood.

EBV is associated with Burkitt lymphoma (BL), Hodgkin disease (HD), and posttransplantation lymphoproliferative disease [7]. These B-cell tumors display distinct patterns of EBV latency gene expression [8]. The oncologic potential of latent EBV is indicated by its unique capacity to growth transform B cells in vitro to lymphoblastoid cell lines (LCLs) [1]. Thus, disruption of latency is not only essential to enable transmission of EBV to other hosts but might be an important factor to limit EBV-induced B-cell lymphoproliferation.

Being immunocompromised increases the risk of EBV-associated B-cell tumors [9]. But nonimmunocompromised individuals may also develop EBV-associated

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BL or HD. EBV-associated HD is more likely to develop when primary EBV infection occurs in adolescence and manifests as infectious mononucleosis with an exuberant immune activation [10]. In endemic BL, >95% of the tumors are EBV positive, and they are epidemiologically linked to *Plasmodium falciparum* malaria, resulting in chronic immune activation [11]. The pattern-recognition receptor Toll-like receptor 9 (TLR9) is abundantly expressed in B cells, and it senses DNA and the malaria parasite's pigment hemozoin [12–14]. We recently demonstrated that TLR9 activation of B cells inhibits lytic EBV during primary infection and inhibits the switching of latent to lytic EBV in chronic infection in vitro [15, 16].

Group A streptococci (GAS) colonize tonsils and the pharynx of up to 20% of healthy children [17]. Considering the high prevalence of EBV and GAS, concomitant presence of both microorganisms in the same individual is frequent. TLR9 senses bacterial DNA and is crucial for controlling GAS infections [18]. One may thus reason that the presence of GAS in tonsils may direct the EBV life cycle toward latency, thereby impairing EBV transmission to other hosts via saliva.

Here, we investigated the influence of GAS on EBV's life cycle and salivary shedding and the mechanisms involved.

METHODS

Ethics Statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. The Ethics Commission of the Canton of Zurich approved the study (StV 40/05). All subjects or their caregivers provided written informed consent.

Cell Culture

The EBV-producer cell line B95.8, Akata BL cells, tonsillar mononuclear cells (TMCs), and LCLs were maintained in Roswell Park Memorial Institute 1640 medium (Sigma-Aldrich, Buchs, Switzerland) with 10% heat-inactivated fetal bovine serum (Life Technologies, Zug, Switzerland), 1% L-glutamine, and 1% penicillin-streptomycin, referred to hereafter as R10.

Enzyme-Linked Immunosorbent Assay (ELISA)

Interleukin 6 (IL-6) or interleukin 10 (IL-10) levels were measured using human IL-6 or IL-10 ELISA kits (R&D Systems, Abingdon, United Kingdom) following the manufacturer's instructions.

DNA Extraction and EBV DNA Detection

Saliva samples were obtained and DNA was extracted as reported elsewhere [19]. EBV DNA levels were determined by quantitative real-time PCR (qPCR) targeting the conserved EBV *Bam*HI W region, as reported elsewhere [20].

RNA Extraction and qPCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. After DNase I treatment, 1 μg of total RNA was used as template for reverse transcription by use of a High-Capacity cDNA Reverse Transcription Kit (Life Technologies). qPCR for human and EBV gene messenger RNA (mRNA) was performed using specific primers and probes for *IL-6*, *IL-10*, or *BZLF1*, as reported elsewhere [15], and for *TLR1–10* (Life Technologies). All reactions were performed on a real-time PCR machine (7900HT; Life Technologies) with TaqMan Gene Expression Master Mix (Life Technologies). The relative gene expression was calculated for each gene of interest by using a $\Delta\Delta C_T$ method, where C_T values were normalized to the value for the housekeeping gene hydroxymethylbilane synthase (*HMBS*) [15].

Isolation of TMCs and EBV Serology

TMCs were isolated from palatine tonsils obtained from patients who underwent routine tonsillectomy, as reported elsewhere [21]. The EBV serologic characteristics of the TMC donors were determined using the Immunodot-Mono G and Mono M kit according to the manufacturer's instructions (Ruwag Diagnostics, Bettlach, Switzerland).

Preparation of Stock EBV

Supernatant of B95.8 cells was obtained as reported elsewhere [21] and stored at −80°C. The cell-free supernatants contained approximately 1×10^8/mL EBV DNA as evaluated by qPCR [20].

GAS Strains

The well-characterized clinical isolate M1T1 GAS strain 5448 [22] was grown to logarithmic phase in Todd-Hewitt broth (Becton Dickinson, Allschwil, Switzerland) containing 2% yeast extract (THY; Oxoid, Pratteln, Switzerland) and was resuspended in Roswell Park Memorial Institute 1640 medium at a final concentration of 2×10^9 colony-forming units/mL. Bacteria were killed by heating (at 85°C for 60 minutes) or by sonication (Sonoplus HD2070; Bandelin Electronic, Berlin, Germany) with 20 kHz at 70 W (amplitude, 100%) for 15 minutes on ice.

Detection of GAS Colonization

Explanted tonsils were rolled over 5% sheep blood agar that was incubated for 48 hours. GAS was identified by latex agglutination for Lancefield group A (Bio-Rad, Cressier, Switzerland) and detection of pyrrolidonyl peptidase production, using L-pyrrolidonyl-beta-naphthylamid (0.7%) disks (Oxoid, Pratteln, Switzerland).

Stimulation of EBV-Infected Cells With GAS

To model acute infection, TMCs plus B95.8 culture supernatants were used, and to model persistent infection, LCLs and Akata cells [23] were used. LCLs were established from TMCs

by infection with B95.8 EBV. Heat-killed or sonicated GAS at a multiplicity of infection (MOI) of 100 or 20 μg/mL of GAS peptidoglycan (Toxin Technology, Sarasota, FL) was added for 24 hours. Apoptosis was assessed using a PE Annexin V Apoptosis Detection Kit I according to the manufacturer's instructions (Becton Dickinson). To neutralize TLR2, LCLs were preincubated with anti-TLR2 polyclonal antibodies (LabForce AG-InvivoGen, Nunningen, Switzerland).

Flow Cytometry

For TLR2 detection, fluorescein isothiocyanate (FITC)–labeled anti-TLR2 monoclonal antibodies (TL2.1; LabForce AG-InvivoGen) were used, and FITC-labeled mouse immunoglobulin G2a (Becton Dickinson) was used as isotype control. For TLR2 and BZLF1 detection, following TLR2 staining, cells were fixed with 2% paraformaldehyde (Sigma-Aldrich) and permeabilized using 0.4% Triton X-100 before adding anti-BZLF1 antibodies (BZ1; Santa Cruz Biotechnology, Heidelberg, Germany) followed by PE-labeled rabbit anti-mouse immunoglobulin G1 (Santa Cruz Biotechnology). Data on apoptosis or TLR2 and/or BZLF1 detection were collected using a FACSCanto II (Becton Dickinson) and were analyzed using FlowJo software.

Statistical Analyses

Analyses of statistical significance were based on a 2-tailed paired or unpaired Student *t* test, Wilcoxon signed rank test, Mann–Whitney *U* test, or χ^2 test. Differences with a *P* value of <.05 were regarded as statistically significant.

RESULTS

Saliva From EBV-Infected GAS-Colonized Individuals Contained More EBV Than Saliva From EBV-Infected Individuals Not Colonized With GAS

We asked whether salivary EBV shedding is influenced by colonization with GAS. Thus, we assayed saliva from EBV-infected individuals who were or were not colonized with GAS by using qPCR targeting EBV DNA to detect viral DNA contained in intact EBV particles. We detected EBV in 7 of 12 GAS-colonized individuals (58%; median age, 6.0 years [range, 2.2–15.0 years]; mean age, 6.2 years) and 9 of 15 GAS-negative individuals (60%; median age, 5.3 years [range, 3.1–13.8 years]; mean age, 6.0 years). The number of EBV DNA copies in saliva from GAS-colonized EBV-infected individuals was higher than that in saliva from noncolonized individuals (*P* = .03; Figure 1).

More-Frequent BZLF1 mRNA Expression in Tonsils From GAS-Colonized Than From Noncolonized EBV-Infected Individuals

The EBV load in saliva may reflect distinct EBV replication in oropharyngeal epithelial cells, mucosa-associated B cells, or both. Since determination of EBV replication in primary epithelial cells is not feasible, we measured *BZLF1* mRNA expression in tonsils. *BZLF1* is an immediate-early lytic EBV gene,

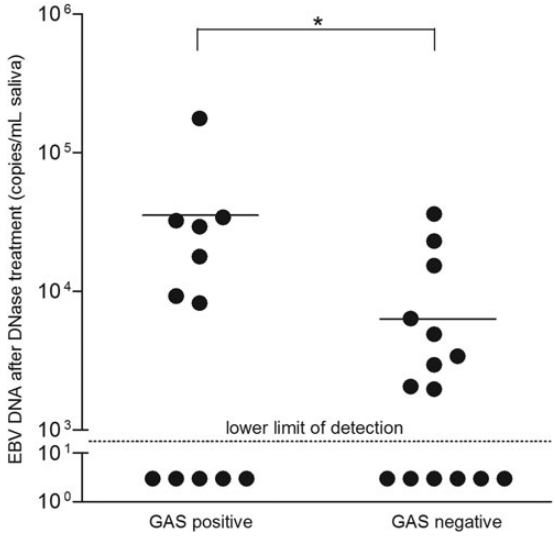

Figure 1. Saliva from Epstein-Barr virus (EBV)–infected group A streptococcus (GAS)–colonized individuals contained more EBV particles than saliva from EBV-infected noncolonized individuals. The number of EBV DNA copies per milliliter in saliva samples from EBV carriers who underwent routine tonsillectomy was determined by quantitative real-time polymerase chain reaction after DNase I treatment. Results for GAS-positive (n = 12) and GAS-negative (n = 15) individuals were compared. *P* values were calculated using the Mann–Whitney *U* test. **P* = .03.

the expression of which is sufficient to initiate EBV replication. Tonsil tissues from 5 of 8 GAS-colonized individuals expressed *BZLF1* mRNA (62.5%; median age, 5.3 years [range, 2.8–6.8 years]; mean age, 4.9 years), in contrast to 1 of 8 GAS-negative individuals (12.5%; median age, 4.8 years [range, 3.1–6.0 years]; mean age, 4.8 years; *P* = .04; Figure 2*A*). IL-6 and IL-10 mRNA expression levels determined by human specific qPCRs showed no significant differences between GAS-positive and GAS-negative tonsils (Figure 2*B* and 2*C*), suggesting a similar inflammation status.

Heat-Killed but Not Sonicated GAS Induced Lytic EBV In Vitro After EBV Exposure

Heat-killed GAS are intact bacteria that mimic colonizing GAS. In contrast, sonication disrupts GAS, resulting in fragmentation and release of bacterial cell wall components and DNA, mimicking an acute GAS infection during which GAS are lysed by both the immune system and antibiotics. We inoculated TMCs with EBV B95.8 and concomitantly stimulated them with heat-killed or sonicated GAS at a MOI of 100. We then measured EBV and *HMBS* mRNA expression levels by qPCR 24 hours after inoculation with EBV. Levels of *BZLF1* mRNA in TMCs 24 hours after stimulation with heat-killed GAS were higher than in nonstimulated TMCs or in TMCs stimulated with sonicated GAS (*P* = .03; Figure 3*A*). Furthermore, heat-killed GAS did not affect latent EBV (LMP1 and EBNA3C; data not

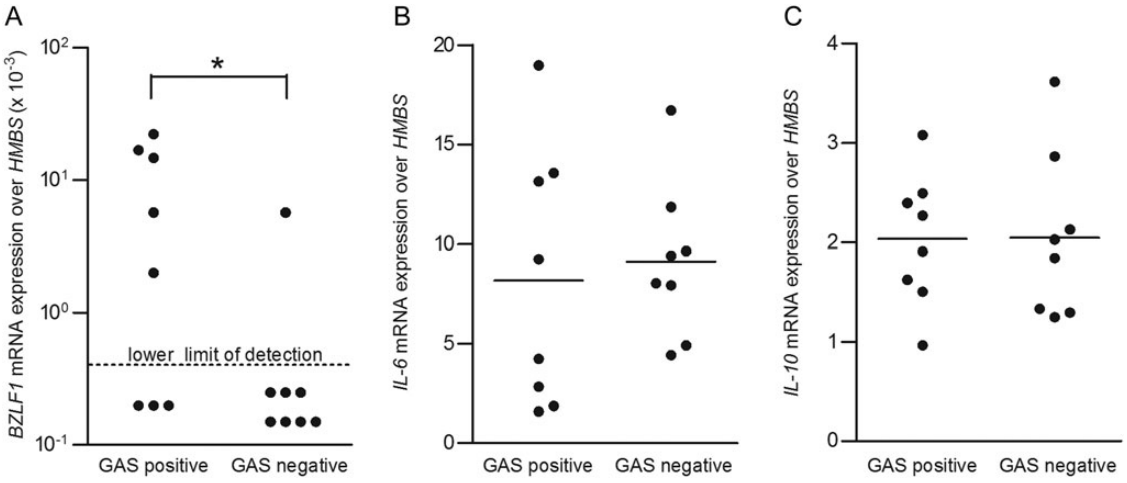

Figure 2. *BZLF1* messenger RNA (mRNA) in tonsils from Epstein-Barr virus (EBV)–infected group A streptococcus (GAS)–colonized individuals was more frequently detectable than in noncolonized individuals. Levels of *BZLF1* (*A*), *IL-6* (*B*), and *IL-10* (*C*) mRNA expression in tonsil tissues from EBV-seropositive patients who underwent routine tonsillectomy were analyzed by quantitative real-time polymerase chain reaction. Results for GAS-positive (n = 8) and GAS-negative (n = 8) individuals were compared. *BZLF1* expression values above the lower detection limit were considered positive, and those below the lower detection limit were considered negative. *P* values were calculated using the χ^2 test. **P* = .04.

shown), IL-6, and IL-10 mRNA expression in TMCs, compared with no stimulus (Figure 3*B* and 3*C*). In contrast, sonicated GAS increased IL-6 and IL-10 mRNA expressions in TMCs, compared with no stimulus or heat-killed GAS ($P = .03$; Figure 3*B* and 3*C*). Thus, neither heat-killed nor sonicated GAS influenced latent EBV gene expression. In contrast, heat-killed GAS strongly induced lytic EBV infection in TMCs exposed de novo to EBV, whereas sonicated GAS did not. The latter induced strong cytokine gene expression in TMCs, whereas heat-killed GAS did not.

Heat-Killed GAS Induced Lytic EBV and Caused Cell Death in Latently EBV-Infected B Cells

Next, we asked whether GAS modulates EBV persistent B-cellular infection. We used LCLs as a model for persistent (latent) EBV infection and stimulated them with heat-killed or sonicated GAS at a MOI of 100. EBV and mRNA expression levels of human genes were measured by qPCR 24 hours later. *BZLF1* mRNA levels in LCLs exposed to heat-killed GAS were higher ($P = .02$) than in LCLs with or without exposure to sonicated GAS (Figure 4*A*).

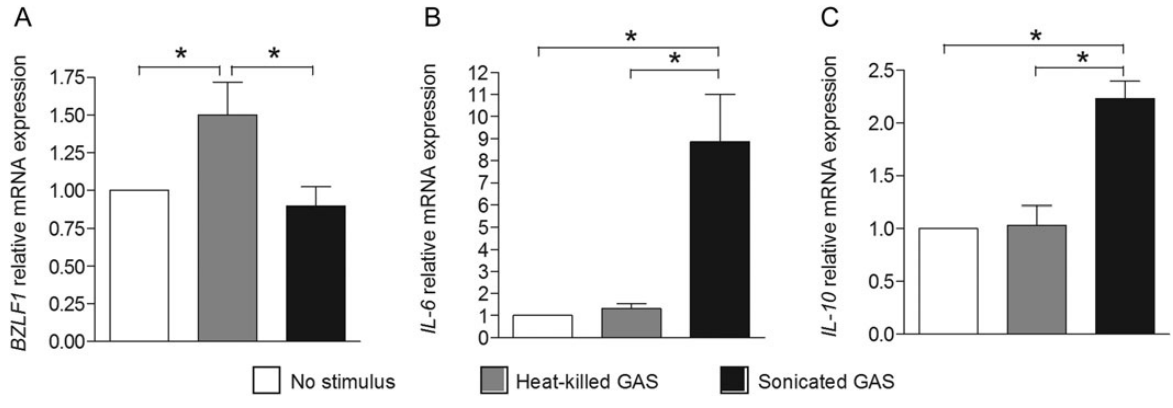

Figure 3. Heat-killed but not sonicated group A streptococcus (GAS) induced lytic Epstein-Barr virus (EBV) after de novo in vitro EBV exposure. Tonsillar mononuclear cells (TMCs) were inoculated with supernatants from B95.8 cells (EBV-producer cells). Concomitantly, heat-killed or sonicated GAS were added, and then cell pellets were harvested and subjected to quantitative real-time polymerase chain reaction to determine expression of *BZLF1* (*A*), *IL-6* (*B*), and *IL-10* (*C*) messenger RNA (mRNA) expression. Results shown were pooled from TMCs from 6 donors and are expressed as mean values ± standard error of the mean. *P* values were calculated using a Wilcoxon signed rank test. **P* = .03.

We asked whether the increased *BZLF1* mRNA levels after exposure to heat-killed GAS were associated with B-cell death. We assessed late apoptosis by determining the percentage of 7-aminoactinomycin D (7-AAD)–stained cells by flow cytometry in LCLs following stimulation or no stimulation. Exposure to heat-killed GAS for 24 hours resulted in higher percentages of 7-AAD–stained cells as compared to exposure to sonicated GAS or controls ($P = .008$; Figure 4*B*). There were no differences in percentages of early apoptotic cells as assessed by flow cytometry after staining with Annexin V and 7-AAD. This was confirmed by assessing cell viability, using the Trypan blue exclusion method (not shown). The number of EBV DNA copies after DNase I treatment in the supernatants of LCLs exposed to heat-killed GAS were higher than in controls ($P = .02$; Figure 4*C*), corroborating our above-described in vivo observations of higher EBV copies in tonsils of GAS-colonized individuals. Exposure to sonicated GAS resulted in lower EBV DNA copies, compared with no exposure ($P = .02$; Figure 4*C*). This is in line with our previous study showing that TLR9 triggering results in suppression of switching from latent to lytic EBV. Sonicated GAS contain increased levels of GAS DNA, which has been recently demonstrated to trigger TLR9 and subsequently induce proinflammatory cytokine expression [18, 24].

Heat-killed GAS did not affect the levels of *EBNA3C* latent EBV (not shown) or *IL-6* (Figure 4*D*), although it slightly upregulated *IL-10* mRNA expression (Figure 4*E*). Similarly, after de novo EBV exposure, sonicated GAS increased *IL-6* ($P = .02$; Figure 4*D*) and *IL-10* mRNA expression in LCLs, compared with no stimulus ($P = .03$; Figure 4*E*), as well as IL-6 and IL-10 protein levels (Figure 4*F* and 4*G*).

We wondered whether heat-killed or sonicated GAS exhibited similar effects on EBV-carrying BL cells as on LCLs. We chose Akata cells because they represent a well-established model for studying the switching from latent to lytic EBV in vitro. We analyzed *BZLF1* mRNA expression 24 hours after treatment with heat-killed or sonicated GAS by qPCR. In contrast to LCLs, levels of *BZLF1* mRNA in Akata cells exposed to heat-killed GAS were not altered, whereas exposure to sonicated GAS significantly decreased endogenous levels of *BZLF1* mRNA expression (Figure 4*H*). These results suggested that sonicated GAS might trigger TLR9. Sonicated GAS samples contain genomic DNA with unmethylated CpG motifs known to trigger TLR9 [18, 24], and triggering of TLR9 results in suppression of lytic induction in Akata cells [15, 16]. No difference in the percentage of 7-AAD stained cells was observed in the cells exposed to heat-killed or sonicated GAS (Figure 4*I*).

Primary B Cells and LCLs Expressed TLR2, in Contrast to Akata Cells

Gram-positive bacteria, such as GAS, are recognized by TLR2 on the cell surface and by TLR9 in endosomes [18, 24]. TLRs are key players in innate immunity and are involved in the recognition of pathogens and microbial products leading to activation of antimicrobial effector pathways [25]. TLR2 generally forms heterodimers with TLR1 or TLR6, which recognize peptidoglycan and lipoteichoic acids from gram-positive bacteria [26]. We asked whether the differences in lytic EBV gene expression were due to distinct expression profiles of TLRs. Thus, we analyzed TLR expression in primary B cells, LCLs, and Akata cells (Figure 5*A*). Primary B cells, LCLs, and Akata cells expressed *TLR1*, *TLR6*, *TLR7*, *TLR9*, and *TLR10* mRNA, whereas *TLR5* mRNA expression was detected at extremely low levels in primary B cells and was not detected in LCLs and Akata cells. *TLR2*, *TLR3*, and *TLR4* mRNA was detected at extremely low levels in Akata cells, in contrast to primary B cells and LCLs. Similar to *TLR2* mRNA, TLR2 protein was expressed in LCLs but not in Akata cells (Figure 5*B*), and the mean fluorescence intensity on LCLs was higher than on Akata cells (Figure 5*C*), as determined by flow cytometry. Since heat-killed Gram-positive bacteria are known TLR2 ligands [27] above results suggested that heat-killed GAS might induce lytic EBV infection in LCLs via TLR2 activation.

Heat-Killed GAS and GAS Peptidoglycan Induce Lytic EBV via TLR2

Exposure of LCLs to either heat-killed GAS, purified GAS peptidoglycan used as control for the GAS cell wall component peptidoglycan, or sonicated GAS did not change the percentages of TLR2-positive cells as determined by flow cytometry (Figure 6*A* and 6*B* upper panel left). Percentages of BZLF1-positive cells in LCLs exposed to heat-killed GAS were higher than in nonexposed LCLs or in LCLs exposed to sonicated GAS (Figure 6*A* and 6*B*), corroborating mRNA expression findings (Figure 4*A*), and exposure to GAS peptidoglycan showed results similar to those for the heat-killed GAS. The proportions of BZLF1-positive cells showed around a 4-fold higher increase among TLR2-positive cells, compared with TLR2-negative cells, after exposure to heat-killed GAS or GAS peptidoglycan (Figure 6*B*). We confirmed that exposure of LCLs to GAS peptidoglycan resulted in similar upregulation of *BZLF1* and *IL-10* mRNA as heat-killed GAS, compared with no exposure (Figure 6*C*). Similar results were obtained using 2 other TLR2 ligands, staphylococcal lipoteichoic acid and staphylococcal peptidoglycan (Supplementary Figure 1) [28, 29]. Finally, anti-TLR2 polyclonal neutralizing antibodies inhibited upregulation of *BZLF1* and *IL-10* mRNA expression (Figure 6*C*) in LCLs exposed to heat-killed GAS or GAS peptidoglycan.

DISCUSSION

We examined the influence of GAS oropharyngeal colonization on EBV's life cycle. We found that (1) EBV carriers shed more EBV particles in their saliva when colonized with GAS, (2)

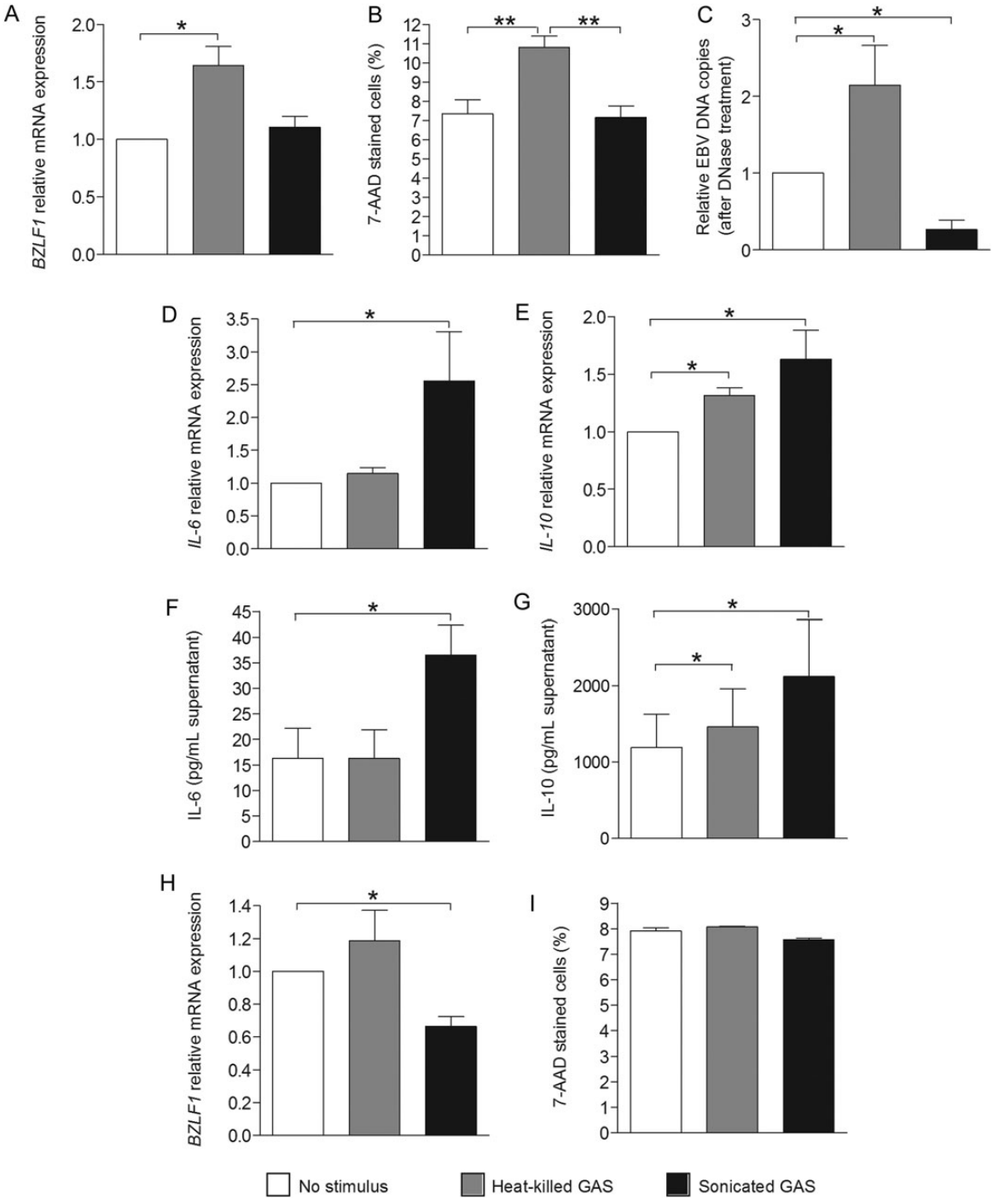

Figure 4. Heat-killed group A streptococcus (GAS) induced lytic Epstein-Barr virus (EBV) and caused increased cell death of latently EBV-infected B cells. Levels of *BZLF1* (*A*), *IL-6* (*D*), and *IL-10* (*E*) messenger RNA (mRNA) expression in lymphoblastoid cell lines (LCLs) were analyzed by quantitative real-time polymerase chain reaction (qPCR) 24 hours after exposure. *B*, 7-aminoactinomycin D (7-AAD)–stained LCLs were analyzed by flow cytometry 24 hours after exposure. The numbers of EBV DNA copies in culture supernatants from LCLs were analyzed by qPCR 24 hours after exposure (*C*). Interleukin 6 (IL-6; *F*) and interleukin 10 (IL-10; *G*) concentrations in supernatants from LCLs were measured by enzyme-linked immunosorbent assay (ELISA). The results shown were pooled from LCLs from 7 donors, from 4 donors (done in duplicate), and from 6 donors for qPCR, flow cytometry, and ELISA, respectively, and are expressed as mean values ± standard error of the mean (SEM). *P* values were calculated by the Wilcoxon signed rank test. **P* < .05, ***P* < .01. *H,* Levels of *BZLF1* mRNA expression in Akata Burkitt lymphoma (BL) cells were analyzed by qPCR 24 hours after exposure to heat-killed or sonicated GAS. *I*, 7-AAD–stained Akata BL cells were analyzed by flow cytometry 24 hours after exposure. Results shown were from Akata BL cells pooled from 3 experiments and are expressed as mean values ± SEM. The *P* value was calculated using a paired Student *t* test. **P* < .05.

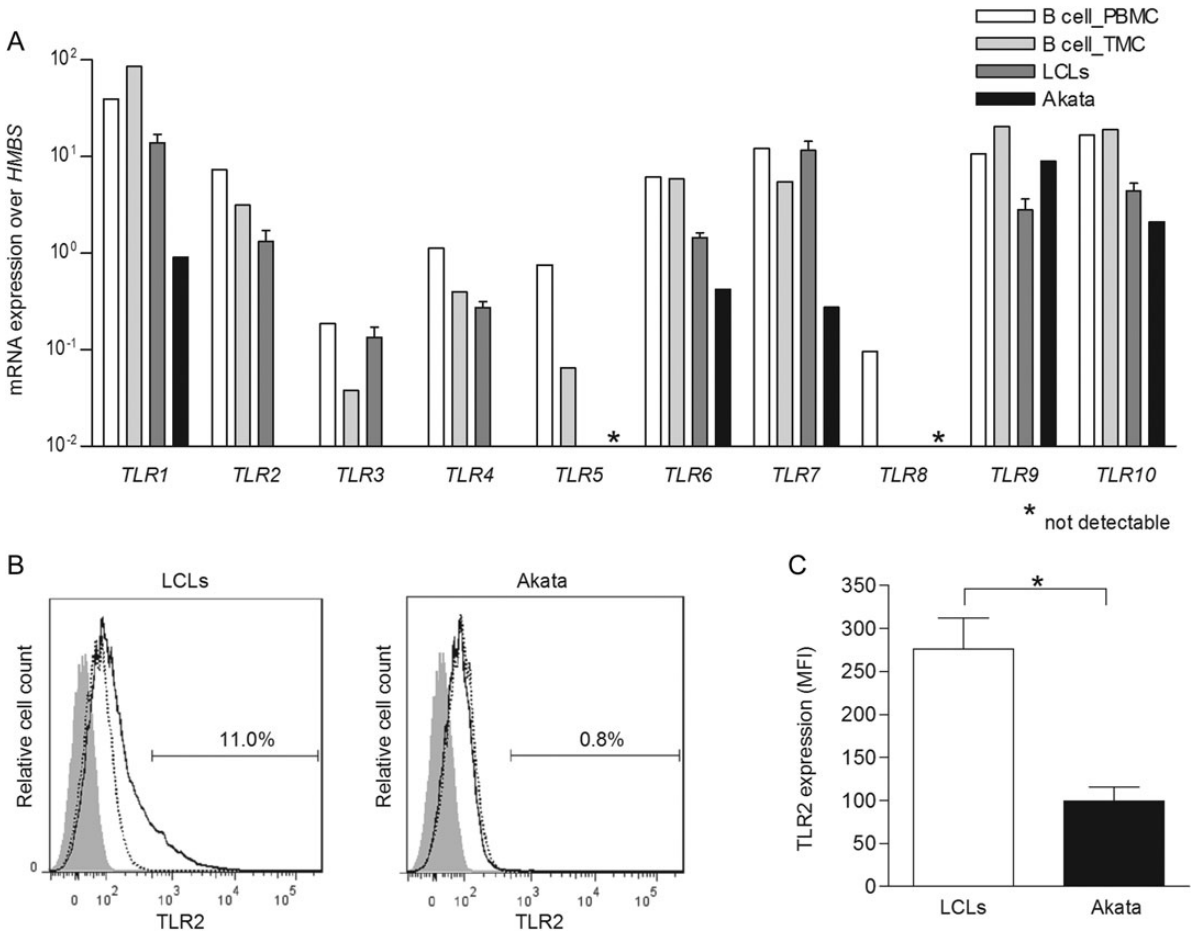

Figure 5. Primary B cells and lymphoblastoid cell lines (LCLs) expressed Toll-like receptor 2 (TLR2), in contrast to Akata BL cells. *A*, Messenger RNA (mRNA) expression levels of *TLR1–TLR10* in peripheral blood and tonsillar primary B cells, LCLs (n = 4), or Akata BL cells were analyzed by quantitative real-time polymerase chain reaction. Representative data of flow cytometry for TLR2 on LCLs and Akata BL cells (*B*) and the mean fluorescence intensity (MFI) from Akata BL cells pooled from 3 experiments and LCLs from 3 donors (*C*) are shown. The closed histogram, dotted line, and solid line represent unstained cells, isotype control, and anti-TLR2 antibody, respectively. The *P* value was calculated using an unpaired Student *t* test. **P* < .05.

EBV's immediate-early lytic gene *BZLF1* mRNA expression was more frequent in tonsils from GAS-positive EBV carriers than from GAS-negative EBV carriers, (3) heat-killed GAS induced lytic EBV in tonsils during primary infection with EBV in vitro and in persistently EBV-infected LCLs resulting in cell death, and (4) heat-killed GAS and purified GAS peptidoglycan induced lytic EBV in LCLs via TLR2 activation. Thus, GAS colonization of the oropharynx might benefit EBV by increasing the salivary EBV load and thereby enhancing the likelihood of EBV spread to other hosts.

Our observation that concomitant GAS colonization resulted in higher salivary EBV shedding is unprecedented. Salivary EBV shedding lasts at least 6 months after primary EBV infection manifesting as infectious mononucleosis [10, 30, 31]. Frequent and abundant salivary EBV shedding is observed in EBV-infected children with tonsillar hypertrophy [19], a condition that is associated with more-frequent GAS colonization [32]. Lytic EBV replication in the oropharynx may occur in epithelial cells, which act as amplifiers [33] after acquiring EBV from tonsillar or other local mucosa-associated B cells [34]. Investigation of lytic EBV replication in oropharyngeal epithelial cells has not been successful. Thus, we assessed the effect of GAS carriage in tonsils from EBV carriers. We found that GAS carriage was associated with significantly higher immediate-early lytic gene *BZLF1* mRNA expression, which induces switching from latent to lytic EBV [1, 6, 35]. In vitro, upregulation of BZLF1 is induced by sodium butyrate [36], 12-O-tetradecanoylphorbol-13-acetate [37], cross-linking of B-cell receptor [38, 39], or the antiinflammatory cytokine transforming growth factor β [40]. Based on IL-6 and IL-10 expression, the inflammation status of tonsils from GAS-colonized individuals did not differ from that of tonsils from noncolonized

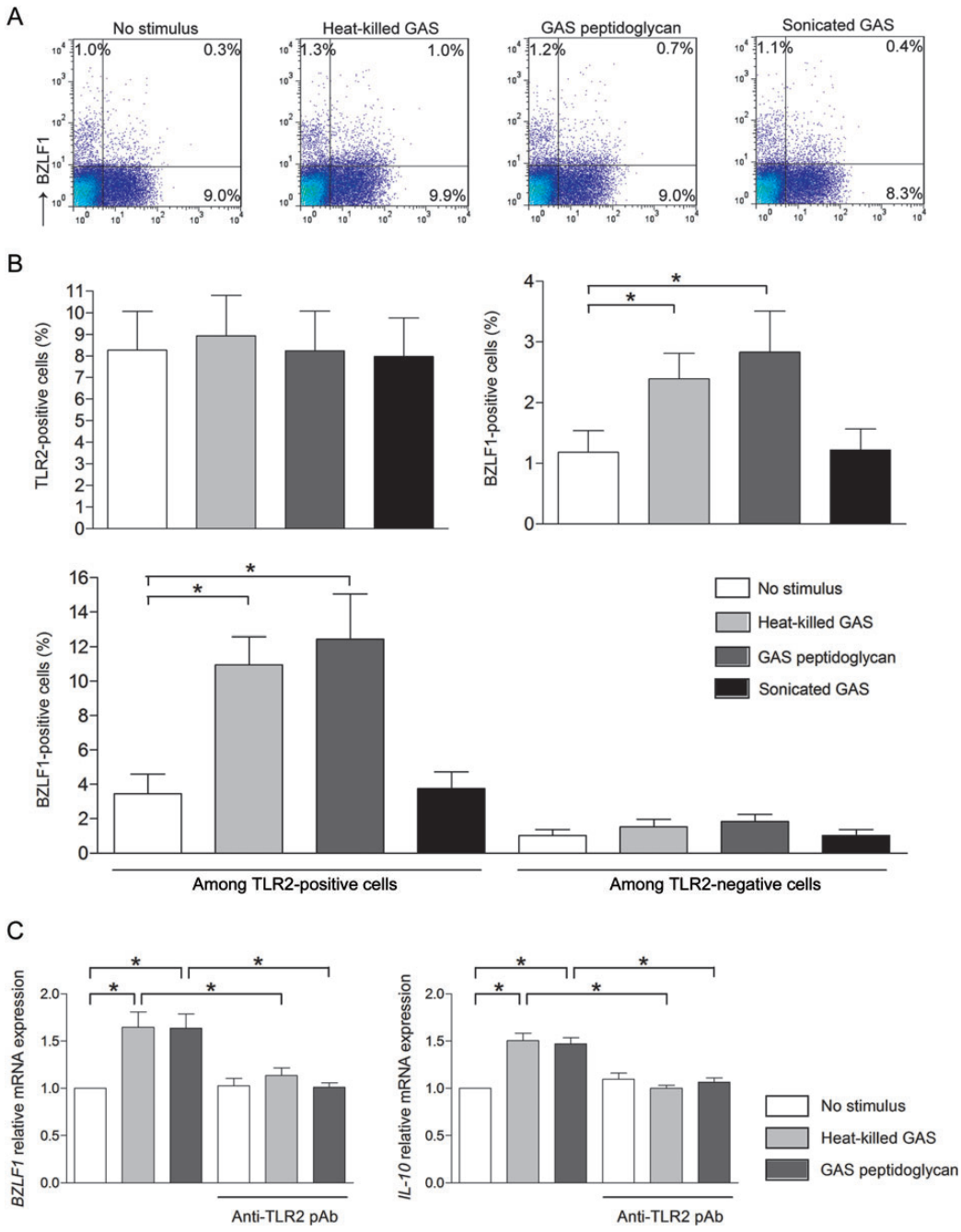

Figure 6. Heat-killed group A streptococcus (GAS) and GAS peptidoglycan induced lytic Epstein-Barr virus via Toll-like receptor 2 (TLR2). *A*, Representative flow cytometry findings for TLR2 and BZLF1 in lymphoblastoid cell lines (LCLs) unstimulated or simulated with heat-killed GAS, GAS peptidoglycan, or sonicated GAS. *B*, Percentages of TLR2-positive cells, BZLF1-positive cells, and BZLF1-positive cells among TLR2-positive cells and TLR2-negative cells following no treatment or treatment with heat-killed GAS or GAS peptidoglycan. *C*, Levels of *BZLF1* and *IL-10* RNA expression in LCLs following no exposure or exposure to heat-killed GAS, GAS peptidoglycan, or sonicated GAS without or with antecedent anti-TLR2 polyclonal neutralizing antibodies (anti-TLR2 pAb) treatment were analyzed by quantitative real-time polymerase chain reaction. Results shown are from LCLs from 6 donors and are expressed as mean values ± standard error of the mean. *P* values were calculated using a Wilcoxon signed rank test. **P* < .05.

individuals in this study. Thus, enhanced EBV lytic replication was not due to a lower proinflammatory or higher antiinflammatory state of the tonsils.

Intriguingly, we found that heat-killed GAS and sonicated GAS exhibited contrasting effects on both EBV and immune activation. Whereas heat-inactivated GAS induced lytic EBV,

sonicated GAS did not. Sonicated GAS, by contrast, increased the expression of IL-6 in TMCs, whereas heat-inactivated GAS did not. Thus, heat-inactivated GAS—intact GAS bacterial cells that may therefore mimic GAS colonization—seem to activate the innate immunity differently from sonicated GAS and have an opposite influence on EBV. GAS DNA found in sonicated GAS suspensions triggers TLR9 and subsequently induces proinflammatory cytokine expression [18, 24]. Activation of Akata cells was diminished when a TLR9 antagonist was added (Supplementary Figure 2). This is compatible with our observation of increased IL-6 expression in TMCs exposed to sonicated GAS but not in TMCs exposed to heat-killed GAS. Importantly, we recently demonstrated that TLR9 triggering suppresses switching from latent to lytic EBV [15, 16].

The upregulation of *BZLF1* following exposure to heat-killed GAS was only approximately 1.5-fold but was highly reproducible, implying that only a small fraction of B cells was provoked to switch to lytic EBV infection. Indeed, we found that around 1% of LCL cells upregulated BZLF1 expression after exposure to heat-killed GAS. An alternative reason for the increase in the level of EBV shedding in GAS-colonized children might be that they have an immunological difference that allows coincident GAS persistence and higher EBV secretion. Nevertheless, no such difference was observed for *IL-6*, *IL-10* (Figure 2*B* and 2*C*), and *TNF-α* mRNA expression levels (Supplementary Figure 3*A*). Surprisingly, heat-killed GAS did not induce switching to lytic EBV in Akata cells, which are commonly used as a model to study the switching from latent to lytic EBV. Thus, we hypothesized that Akata cells may differ from primary B cells and LCLs in their expression of TLRs. Indeed, we found that Akata cells expressed TLR2 at remarkably lower levels, if at all, than primary B cells and LCLs. Exposure of LCLs to GAS peptidoglycan, a known ligand for TLR2, provoked similar expression of BZLF1 as heat-killed GAS. Upregulation of BZLF1 was around 4-fold higher in TLR2-positive B cells, compared with TLR2-negative B cells. Importantly, anti-TLR2 neutralizing antibodies completely abrogated upregulation of *BZLF1* following exposure to heat-killed GAS or GAS peptidoglycan. This strongly suggested that heat-killed GAS and GAS peptidoglycan upregulated BZLF1 expression in LCLs via TLR2 triggering. Finally, we showed that other known TLR2 ligands, namely staphylococcal lipoteichoic acid and peptidoglycan [28, 29], also induced *BZLF1* mRNA expression in LCLs, suggesting the engagement of TLR2 results in lytic reactivation of EBV.

Our novel findings imply that TLR2 triggering may disrupt EBV's default state of latency. This seems to be beneficial for EBV since it allows EBV to spread to other hosts. However, it may put EBV latency and the survival of the host cell at stake. In view that TLR2-expressing EBV-infected cells are likely to undergo switching to lytic EBV, selection of EBV-infected B-cells subsets with lower or no expression of TLR2 may take place over time. Notably, we found that *TLR2* mRNA levels in EBV-negative BL cells were at least 5-fold higher than in EBV-positive BL cells (Supplementary Figure 3*B*), suggesting that cells expressing low TLR2 levels may have been more likely to survive than cells expressing higher levels of TLR2. Alternatively, EBV may induce downregulation of TLR2 expression, thereby preventing switching to lytic infection and securing its latent state. Indeed, the expression of *TLR2* in tonsillar B cells decreased in a time-dependent fashion after inoculation with EBV (Supplementary Figure 3*C*). Thus, TLR2 signal transduction may contribute to balancing latent and lytic EBV infection.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflict of interest. All authors: No reports conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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A Distinct Subpopulation of Human NK Cells Restricts B Cell Transformation by EBV

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NK cells constitute the first line of defense against pathogens and transformed cells. They mature in secondary lymphoid organs, including tonsils, where common pathogens, such as EBV, enter the host and potentially imprint differentiating cells, which then patrol the body via the blood stream. Therefore, we set out to characterize a distinct human NK cell population in tonsils that produces high amounts of the immunomodulatory and antiviral cytokine IFN-γ. We found that the tonsilar IFN-γ^{high} NK cell subset is $CD56^{bright}NKG2A^{+}CD94^{+}CD54^{+}CD62L^{-}$, is present in tonsils ex vivo and is more mature than other $CD56^{bright}$ NK cells in tonsils and less mature than other NK cells in blood, shows very low plasticity even after prolonged cytokine stimulation, accumulates in tonsils of EBV carriers, and is able to potently restrict EBV-induced transformation of B cells. Thus, we characterized a distinct and stable IFN-γ^{high} NK cell subpopulation that can specifically restrict malignant transformation of EBV-infected B cells. This subset should be exploited for future development of cell-based therapeutic approaches in EBV-associated malignancies. ***The Journal of Immunology*****, 2013, 191: 4989–4995.**

Natural killer cells are innate lymphocytes that were originally described for their ability to target virus-infected and transformed cells without preactivation (1, 2). Although they originally were thought to leave the bone marrow as fully developed effector cells and to reside primarily in the blood stream, reservoirs of NK cells were discovered more recently in other tissues, including secondary lymphoid organs (SLOs) (3, 4). These NK cells in lymph nodes and tonsils (SLOs) require activation by primarily dendritic cells to achieve full functional competence and mediate protection against pathogens (4–6). In tonsils, in contrast to peripheral blood, the most mature NK cells are $CD56^{bright}$, whereas earlier differentiation stages are $CD56^{dim}$ (7). $CD56^{+}$ cells in human tonsils also contain $ROR\gamma t^{+}$ ILC3 cells (8, 9); for a more detailed characterization of $CD56^{dim}$ tonsilar cells, these cells would need to be taken into account. In humans, the NK cell reservoir in SLOs consists mainly of $CD56^{bright}CD16^{-}$ NK cells. In peripheral blood, NK cells of this subset carry homing markers to SLOs and constitute only about one tenth of the circulating NK cells (4, 10). These blood $CD56^{bright}$ NK cells have longer telomeres and can develop into functional $CD56^{dim}CD16^{+}$ NK cells (4, 11, 12). Subsequently, they seem to acquire more and more inhibitory receptors, including killer Ig–like receptors (KIRs), during further differentiation (13). However, in contrast to mouse NK cells, which excel in their main effector functions (i.e., cytokine production and cytotoxicity) only after terminal differentiation (14), human NK cells acquire already potent cytokine production at the $CD56^{bright}CD16^{-}$ differentiation stage (10). Intriguingly, NK cells in SLOs produce much more IFN-γ in response to IL-12 than do peripheral blood NK cells, and this reactivity resides nearly exclusively in the $CD56^{bright}CD16^{-}$ NK cells of these organs (15). This effector function of NK cells can be especially useful in secondary lymphoid tissues at mucosal surfaces (e.g., tonsils) for restricting infections, because these are the sites where pathogens are first encountered as a natural entrance point.

One such pathogen, which could be targeted by NK cell responses in tonsils, is EBV. EBV is a common γ-herpesvirus that infects >90% of the human adult population and enters the human body via tonsils after transmission in saliva (16). In the vast majority of infected individuals, EBV persists without symptoms under tight immune control by primarily T cells (17). However, in a subset of EBV carriers, the B and epithelial cell–transforming capacity of the virus causes detrimental EBV-associated malignancies (18). In this respect, the initial immune response to infection seems to be quite decisive, because symptomatic primary EBV infection (infectious mononucleosis) can predispose for EBV-associated malignancies (19, 20) and even autoimmune diseases (21). NK cells are part of this innate immune response to EBV and have been reported, somewhat controversially, to correlate either inversely or directly with viral titers during primary infection (22, 23). A protective function of these NK cell responses during primary infection is

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A.L. developed, planned, and performed the experiments; analyzed the data; wrote the manuscript; and prepared the figures. L.D.V. provided expert technical help. T.A. provided PBMC samples from age-matched pediatric tonsil donors. D.N. provided tonsil and matched blood samples and commented on the manuscript. C.M. developed, helped plan, and procured funding for the research; and wrote the manuscript.

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Abbreviations used in this article: FSC/SSC, forward/sideward scatter; IR, immunoresponsive; KIR, killer Ig–like receptor; MFI, median fluorescence intensity; rh, recombinant human; SLO, secondary lymphoid organ.

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indicated by primary immunodeficiencies that affect NK cells and were suggested to predispose for EBV-associated malignancies (24, 25). Furthermore, X-linked lymphoproliferative disease–associated mutations, which predispose affected males to fatal lymphoproliferative disease upon primary EBV infection, compromise NK cell recognition of EBV-transformed cell lines (26). These mutations also affect other components of cell-mediated immunity, such as T cells. Nevertheless, NK cells clearly contribute to early restriction of EBV infection and lower the risk for EBV-associated diseases. Palatine tonsilar NK cells, which are located at the site of EBV entry, are ideally suited to mediate this early restriction, and we demonstrated previously that NK cells of these SLOs can limit B cell transformation by EBV with their superior ability to produce IFN-γ (15). In the same study, we also noted that only a subset of tonsilar NK cells produced these high levels of IFN-γ. Therefore, in this study we set out to characterize this IFN-γ^{high} NK cell subset.

In this article, we report that cells from this IFN-γ^{high} NK cell subset are $CD56^{bright}NKG2A^+CD94^+CD54^+CD62L^-$, are enriched in tonsils of EBV carriers, and specifically restrict EBV transformation of B cells. Moreover, this subpopulation lacks homing markers to SLOs and seems to be primed to transition into the periphery to carry out innate immune control of EBV infection.

Materials and Methods

Ethical approval for human samples

Studies on human samples for the immune control of human γ-herpesvirus infections were reviewed and approved by the cantonal ethical committee of Zürich, Switzerland (KEK-StV-Nr. 19/08). Tonsils and blood samples of children were collected after informed consent of the legal guardians. Lymphocyte concentrates from adult donors were obtained from the Swiss Red Cross Blutspendedienst Zürich (Zürich, Switzerland) after informed consent of the donors.

Reagents

The following anti-human mAbs were used in this study: CD3 Pacific Blue (Invitrogen); CD3 allophycocyanin Cy7, CD56 PE Cy7, IFN-γ allophycocyanin, CD19 PE, CD19 allophycocyanin Cy7, CD94 PE, CD62L allophycocyanin, CD62L PE, CD21 allophycocyanin, CD23 FITC, CD336 PE, CD103 FITC, and isotypes (BD Biosciences); CD117 PE (Miltenyi Biotec); NKG2C allophycocyanin (R&D Systems); CD45RA FITC and CCR7 PE, CD57 FITC, CXCR3 allophycocyanin, perforin FITC, CD54 Pacific Blue, and CD16 allophycocyanin Cy7 (BioLegend); NKG2A PE, NKG2A allophycocyanin, and KIRs—CD158a,h PE, CD158b1,b2,j PE, CD158a,h allophycocyanin, and CD158b1,b2,j allophycocyanin—(Beckman Coulter). Live cells were routinely distinguished using the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen). The following endotoxin-free recombinant human (rh) cytokines were used: rhIL-12 (R&D Systems), rhIL-2 (PeproTech), and rhIL-15 (Sigma).

NK and B cell purification and cell culture

Tonsilar cell single-cell suspensions were obtained by mechanical dissociation of the tissue and mechanical filtration through a 75-μm nylon cell strainer. After Ficoll-Paque Premium gradient centrifugation (GE Healthcare) to remove dead cells and debris, cells were washed extensively and partly cryopreserved or used directly. Leukocytes of blood samples and leukocyte concentrates were purified by Ficoll Paque Premium gradient centrifugation and washed several times. B cells were isolated through magnetic bead cell separation (MACS; Miltenyi Biotec) by $CD19^+$ selection. NK cells were isolated through negative MACS selection using the NK cell isolation kit (Miltenyi Biotec), or $CD19^-$ cells were flow sorted on an Aria III cell sorter (BD Biosciences) through an 85-μm nozzle for purity. Purity was consistently >95%.

For intracellular IFN-γ detection, NK cells were incubated for 18 h with 10 ng/ml rhIL-12, and a final concentration of 2 μM monensin was added (1000× solution; eBioscience) for the last 6 h. Cells were then harvested, washed with PBS and, after staining with the surface-binding Abs, fixed with 2% paraformaldehyde, and intracellular staining was performed in PBS plus 1% Saponin (Fluka) and BSA 1% (Sigma) using anti–IFN-γ Abs (clone 4S.B3; BD Biosciences).

Preparation of EBV

Wild-type EBV was produced through standard 4-h METAFECTENE PRO (Lucerna) transfection of the B95-8 bacmid carrying 293HEK cells in 10-cm dishes, according to the manufacturer's protocol. In brief, plasmids (*BZLF-1*, Amp resistance; and *BALF-4*, gp110, Amp resistance) were preincubated with METAFECTENE at room temperature for 20 min and then added to the cell dishes for 4 h in antibiotic-free medium. Then, RPMI 1640 + 10% FCS medium was replaced with gentamicin-containing RPMI 1640 + 10% FCS medium medium. Supernatant was harvested after 3 d and filtered through 0.45-μm filters, and EBV was further purified and concentrated by ultracentrifugation at 30,000 × *g* for 2 h at 4°C, resuspended in RPMI 1640, and titered on Raji cells. GFP^+ cells were counted 2 d later, and titers were calculated in Raji infectious units.

B cell–transformation assay

This assay was adapted from the previously described protocol (15). Briefly, MACS-isolated B cells (as described above) were plated at 1×10^5 cells and cultured in RPMI 1640 + 5% human serum (Invitrogen) with 2 μg/ml gentamicin and infected with wild-type EBV; sorted NK cells were added at the indicated numbers. IL-12 was added at 10 ng/ml. Cells were harvested after 12 d, and the numbers of transformed B cells were quantified by determining the ratio of live $CD19^+CD21^+CD23^+$ transformed B cells/total live cells by flow cytometry. Percentage of restriction of B cell transformation = (1 − total transformed B cell number of sample with NK cells/total transformed B cell number of sample without NK cells) × 100.

Flow cytometry and data analysis

Flow cytometric data were acquired on an LSR II Fortessa cell analyzer (equipped with four lasers; Figs. 1, 2, 4) or an Aria III cell sorter (five laser lines; Fig. 3) (both using Diva software; all from BD Biosciences). General gating strategy for NK cells: lymphocyte population in forward/sideward scatter (FSC/SSC), live ($Aqua^-$) cells, $CD3^-$, $CD56^+$. Data were analyzed using FlowJo 9.3.1.3. and GraphPad Prism for Mac version 5.0a.

Statistical analysis

Data were compared using the two-tailed Wilcoxon signed-rank test, unless noted otherwise, or the two-tailed Mann–Whitney *U* test, if specified in the figure legends. A p value ≤ 0.05 was considered statistically significant. Error bars represent the SEM in all figures.

Results

The tonsilar immunoresponsive NK cell subset with high IFN-γ production consists of $CD56^{bright}NKG2A^+CD94^+CD54^+CD62L^-$ cells

Tonsils harbor a distinct subset of very high IFN-γ–producing $CD56^{bright}$ NK cells, which secrete this cytokine especially after IL-12 stimulation (4). This subset is not seen in peripheral blood, might play important bridging functions with the adaptive immune system, and could be an adaptation to target pathogens in SLOs at mucosal entry sites. To characterize this subset in more detail, we first determined its surface marker phenotype. To accomplish this, we purified NK cells by negative magnetic cell separation (MACS; Miltenyi Biotec) and stimulated them with 10 ng/ml IL-12 for 18 h. IFN-γ^{high} NK cells differ from the total $CD56^{bright}$ NK cells by their expression of NKG2A/CD94 and CD54, as well as the lack of L-selectin (CD62L) (Fig. 1A–C, Supplemental Fig. 1 [representative flow cytometry dot blots of subpopulations]). IL-12 was chosen for the stimulation of tonsilar NK cells, because it was previously identified as the primary monokine involved in NK cell activation by dendritic cells (15). To exclude the possibility that CD62L undergoes shedding and is lost from the surface of the NK cells due to sample preparation we determined its expression on naive T cells versus T EMRA cells (effector memory RA^+) and found normal levels of L-selectin expression (Supplemental Fig. 3B). Tonsilar IR cells expressed slightly less NKG2C than did other $CD56^{bright}$ NK cells, but the overall expression was very low (data not shown). We confirmed the phenotype of tonsilar NK cells with high IFN-γ production by gating on $CD56^{bright}NKG2A^+CD94^+CD54^+CD62L^-$

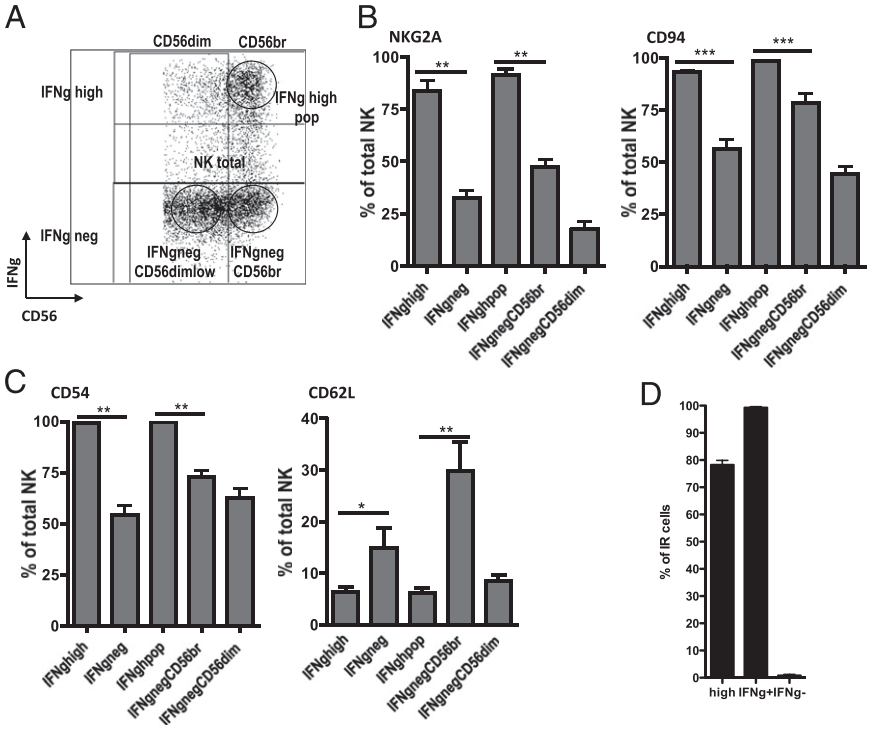

FIGURE 1. The human tonsilar IFN-γ^{high} NK cell IR subset is characterized as CD56^{bright} NKG2A$^+$CD94$^+$CD54$^+$CD62L$^-$. Purified NK cells were stimulated with 10 ng/ml IL-12; monensin was added after 12 h, and cells were analyzed after 18 h by flow cytometry. (**A**) Gating strategy for analysis of distinct IFN-γ^{high} NK cell subset after IL-12 stimulation. The IFN-γ^{high} subset in tonsils is CD56^{bright} NKG2A$^+$CD94$^+$ (**B**) and CD54$^+$CD62L$^-$ (**C**). (**D**) Gated on this subset, these NK cells are almost exclusively IFN-γ^+ after IL-12 stimulation (10 ng/ml, as above). Gating strategy: lymphocyte population in FSC/SSC, Live cells (Live/Dead cell stain Aqua$^-$), NK cells (CD3$^-$ CD56$^+$), subset gating as in (A). At least three independent experiments were performed, and data were summarized from >10 healthy donors. All error bars represent SEM. *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$ two-tailed nonparametric Mann–Whitney *U* test.

cells after 18 h of IL-12 stimulation (10 ng/ml, as above) and found that this population consisted of nearly all IFN-γ–producing cells (Fig. 1D). To exclude overlap with the recently described tonsilar subset of CD336$^+$CD103$^+$ cells (27), we analyzed coexpression with these markers, as well as IFN-γ production, and found that both subsets were distinct (Supplemental Fig. 2). Taken together, these data show that IFN-γ^{high} NK cells in tonsils can be defined as CD56^{bright}NKG2A$^+$CD94$^+$CD54$^+$CD62L$^-$.

IL-12 induces accumulation of high IFN-γ–producing NK cells in tonsils, which are more mature than the other tonsilar NK cells but less mature than peripheral blood NK cells

Next, we addressed whether this distinct NK cell subset (CD56^{bright} NKG2A$^+$CD94$^+$CD54$^+$CD62L$^-$) is already present in tonsils without stimulation. To determine this, we analyzed tonsilar cells from children ex vivo and found a significant population of this subset (Fig. 2A). This subpopulation accumulated after stimulation of purified NK cells with IL-12 for 18 h (Fig. 2A). Expression of c-kit (CD117) is lost throughout the maturation process of NK cells (7); hence, its expression characterizes less mature cells. We found that fewer immunoresponsive (IR) NK cells express c-kit in tonsils compared with the other CD56^{bright} NK cells in this organ. Conversely, more NK cells with this phenotype displayed this marker of immaturity in the peripheral blood compared with the remaining blood CD56^{bright} NK cells (Fig. 2B, Supplemental Fig. 3A [gating strategy]). As a second parameter of maturation for NK cells, we determined the expression of KIRs, which are acquired throughout the maturation process (7). We found that IR NK cells express significantly more KIRs than do tonsilar CD56^{bright} NK cells in general (Fig. 2C). In addition, we found that maturation-associated CD16 expression was significantly higher on tonsilar IR cells than on the rest of CD56^{bright} cells in tonsils, whereas this expression pattern is reversed in blood (i.e., blood IR cells carry less CD16 than do the other peripheral CD56^{bright} cells [Fig. 2G, 2H]). Overall, CD57 and perforin expression was very low, and there was no significant difference between the analyzed subsets (Supplemental Fig. 3C, 3D). As shown in Fig. 2E and 2F, we did not detect any difference in CXCR3 expression between IR and the rest of the CD56^{bright} subset, nor in NKG2A median fluorescence intensity (MFI) on cells pregated on NKG2A$^+$ cells, which might have further supported the advanced maturation status of these cells. In conclusion, we demonstrated that the distinct IR NK cell subset is already present in tonsils and blood ex vivo, accumulates upon IL-12 stimulation, and is more mature than the total tonsilar CD56^{bright} NK cells but less mature than blood NK cells.

The IR NK cell subset shows very low plasticity even after prolonged cytokine stimulation

Plasticity of immunological receptors determines whether molecules are reliable and characteristic marker molecules, defining the same cell subset under different activation conditions. High plasticity will hinder traceability of subsets through different conditions. Therefore, we assessed whether expression of the defined potential marker molecules was stable, even during prolonged cytokine stimulation, to determine whether this subset is stable throughout immunological processes and activation. The subset was sorted into the IR subset, the rest of CD56^{bright} and the CD56^{dim} NK cells (subset gating strategy in Fig. 3A) and stimulated for 7 d with IL-12 (10 ng/ml), IL-2 (100 U/ml), or IL-15 (100 pg/ml). Cells were collected, and expressions of marker molecules were analyzed. CD56 expression was highly variable after cytokine stimulation with IL-12 in our experimental setting (Supplemental Fig. 4A) and with IL-2 and IL-15 (Supplemental Fig. 4B, 4C). The IR subset became largely CD56^{dim} after prolonged cytokine stimulation, which suggested a maturation status similar to blood CD56^{bright} NK cells that were reported to turn into CD56^{dim} upon stimulation (11). The other CD56^{bright} cells conserved their high CD56 expression. CD56^{dim} NK cells, which are the less mature NK cell population in tonsils, turned into CD56^{bright} cells upon prolonged IL-2 stimulation. Thus, CD56 expression was disregarded for reanalysis of the potential marker molecules NKG2A and absence of CD62L. Expression of NKG2A was highly stable (Fig. 3B), and L-selectin negativity was preserved (Fig. 3C), even after prolonged cytokine

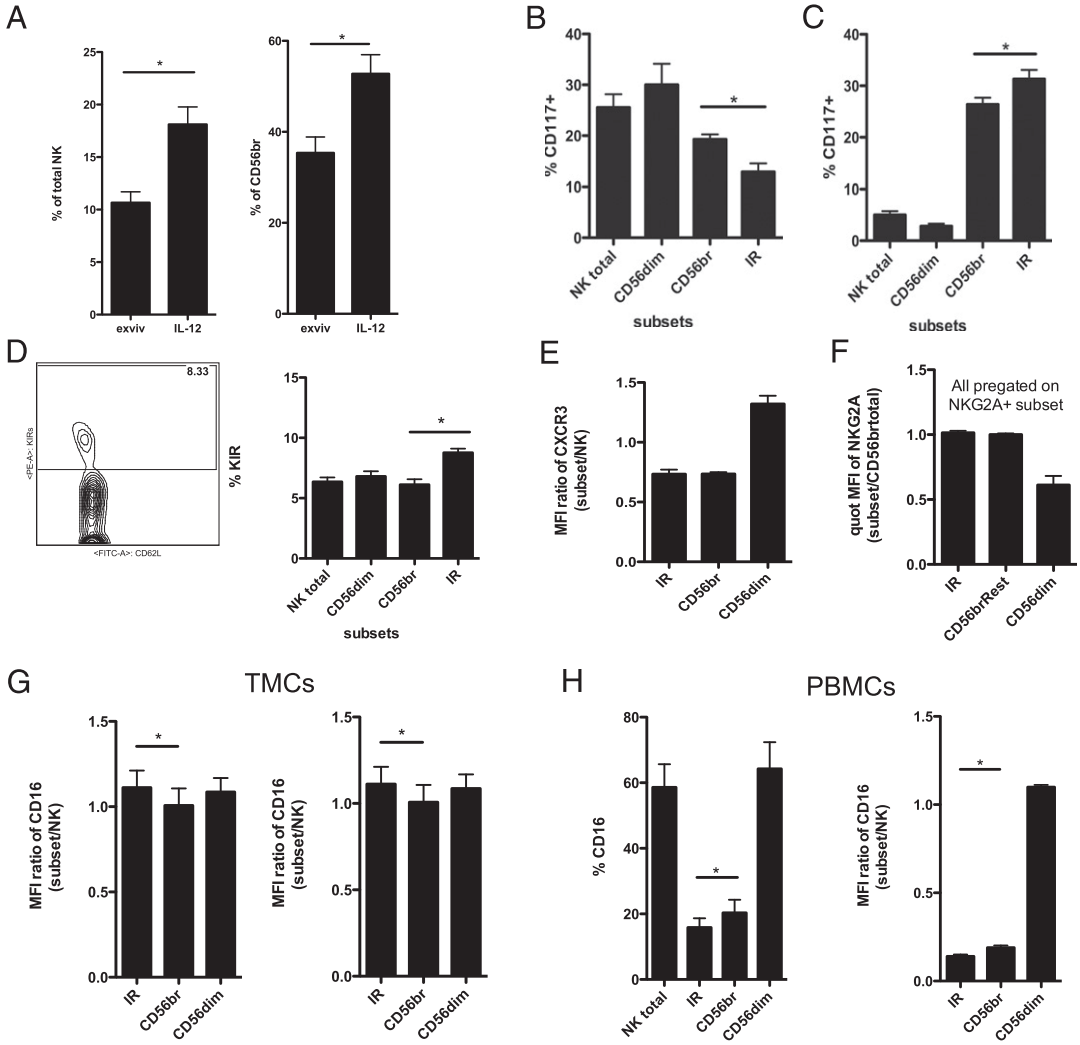

FIGURE 2. The NK cell IR subset is present in tonsils ex vivo and accumulates upon stimulation with IL-12. (**A**) Percentages of total NK cells in the tonsil and CD56^{bright} cells. Subset accumulates after 18-h stimulation with IL-12 (10 ng/ml). Significantly fewer tonsilar IR cells express c-kit (CD117) (**B**), whereas significantly more blood IR cells express this molecule (**C**), which is expressed by more immature NK cells. (**D**) More of the tonsilar IR subset expresses KIR molecules compared with total or CD56^{bright} NK cells. IR cells express similar levels of CXCR3 (**E**) and the NKG2A MFI (subset versus CD56^{bright} total) was not different between IR cells and the other CD56^{bright} cells (**F**), pregated on NKG2A^{+} cells. (**G**) Greater frequency of CD16^{+} IR cells than CD16 expression in other CD56^{bright} cells in tonsilar mononuclear cells (TMCs) and a higher level of expression (as measured by MFI ratio [subset/NK]). (**H**) In contrast, in PBMCs, a higher frequency of the other CD56^{bright} cells expressed CD16 than did IR cells and to a greater extent, as measured by MFI ratio (subset/NK). Gating strategy: lymphocyte population in FSC/SSC, Live cells (Live/Dead Cell Stain Aqua^{-}), NK cells (CD3^{-}CD56^{+}), subset gating: CD56^{bright}NKG2A^{+}CD94^{+}CD54^{+}CD62L^{-}, as depicted in Supplemental Fig. 3A. Data represent at least three independent experiments and at least three donors. All error bars represent SEM. *$p \leq 0.05$.

stimulation with different cytokines in the IR subset. To identify the origin of the IR cells that accumulated after cytokine stimulation, expression of NKG2A (Supplemental Fig. 4C) and the absence of L-selectin (Supplemental Fig. 4D) and of the combined phenotype (Supplemental Fig. 4E) were analyzed on the rest of the initially CD56^{bright} and CD56^{dim} subset after prolonged cytokine stimulation. There was significant acquisition of NKG2A, loss of CD62L, and acquisition of the combined phenotype in CD56^{dim} and the other CD56^{bright} NK cells as the result of IL-2 and IL-12 stimulation. After prolonged cytokine stimulation of purified tonsilar NK cells, the IR subset retained the ability for elevated IFN-γ production (Fig. 3D). In conclusion, the defined potential marker molecules could be confirmed for the IR subset because they are preserved and stable throughout immunological activation modeled by prolonged cytokine stimulation; therefore, they define the subset reliably and characteristically under different activation conditions and can be acquired by non-IR tonsilar NK cells.

IR NK cells accumulate in tonsils of EBV carriers and are able to restrict EBV-induced transformation of B cells

To investigate whether the defined IR NK cell subsets could provide a barrier function against pathogens that infect via the tonsils, we investigated their enrichment in EBV-seropositive individuals. Tonsils of children carrying EBV harbored significantly more of the IR subset of NK cells than did those of age-matched children not infected with EBV (Fig. 4A). The IR subset exists in peripheral blood ex vivo, even though this subset is markedly enriched in tonsils, and there are significantly fewer cells of this subset in the peripheral blood of EBV-infected children than in age-matched noninfected children. Adults have even fewer of the IR subset than do children (Fig. 4B). The IR cells in the peripheral blood have a higher capacity for IFN-γ production after IL-12 stimulation than do the other CD56^{bright} NK cells (Supplemental Fig. 4G). The elevated frequency of IFN-γ–producing IR NK cells, as gated in Fig. 1A, in EBV^{+} tonsils was increased further after IL-12

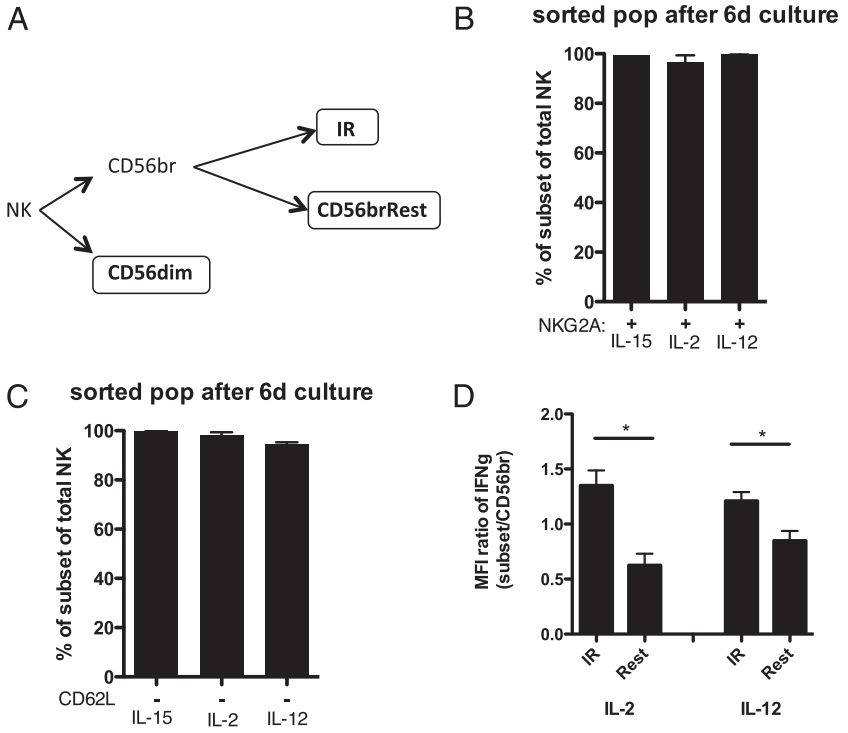

FIGURE 3. The NK cell IR subset shows low plasticity, even after prolonged cytokine stimulation. NK cells were sorted into IR cells, other CD56^{bright} NK cells, and CD56^{dim} NK cells and stimulated with IL-15 (100 pg/ml), IL-12 (10 ng/ml), or IL-2 (100 U/ml) for 6 d, and NKG2A positivity and CD62L negativity were analyzed. CD56 expression changed after prolonged stimulation, as shown in Supplemental Fig. 4. (**A**) Sorting strategy; circled subsets are compared. Regardless of CD56 expression, IR subset shows very low plasticity in expression of NKG2A positivity (**B**) and CD62L negativity (**C**), even after prolonged stimulation with cytokines. (**D**) IR cells retain their superior ability to produce IFN-γ compared with the other CD56^{bright} cells, as shown by the MFI ratio (subset/NK) after prolonged stimulation with IL-12 or IL-2. General gating strategy: lymphocyte population in FSC/SSC, live cells (Live/Dead Cell Stain Aqua^{-}), NK cells (CD3^{-}CD56^{+}), IR: CD56^{bright}NKG2A^{+}CD94^{+}CD54^{+}CD62L^{-}, as depicted in Supplemental Fig. 3A. Furthermore, subset gating for sorting was performed as in Fig. 1A, and for the analysis after cytokine stimulation, both NKG2A and CD62L were assessed. Data represent at least three independent experiments, with a total of three to six donors. All error bars represent SEM. *$p \leq 0.05$.

stimulation (i.e., 1.6-fold in EBV^{-} donors and 1.25-fold in EBV^{+} donors) (Fig. 4C). To characterize whether this accumulation of IR

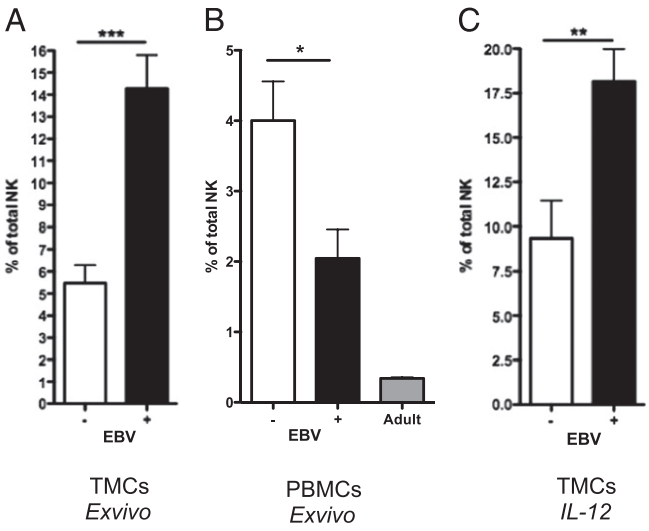

FIGURE 4. IR NK cells accumulate in EBV-infected tonsils. (**A**) Tonsils of children carrying EBV, a chronic and cancerogenic virus infection entering the human body via the tonsils, harbor significantly more of this subset of NK cells than do noninfected children. (**B**) IR subset exists in peripheral blood ex vivo, even though this subset is enriched in tonsils and there is significantly less of this subset in the blood of EBV-carrying children than in noninfected children. Adults have even less of the IR subset than do children. (**C**) Increased numbers of IR cells in EBV^{+} tonsils after IL-12 stimulation (i.e., mean frequency 1.6-fold in EBV^{-} donors and 1.25-fold in EBV^{+} donors). Gating strategy: lymphocyte population in FSC/SSC, Live cells (Live/Dead Cell Stain Aqua^{-}), NK cells (CD3^{-}CD56^{+}), subset gating CD56^{bright}NKG2A^{+}CD94^{+}CD54^{+}CD62L^{-}, as depicted in Supplemental Fig. 3A. Data represent at least three independent experiments, with a total of 4–12 donors. All error bars represent SEM. *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$ two-tailed nonparametric Mann–Whitney *U* test.

NK cells could provide protection in tonsils of EBV-seropositive individuals, we tested this NK cell subset's ability to restrict EBV infection. EBV restriction by NK cells was measured by the B cell–regression assay, as described previously (15). Briefly, 100,000 MACS-sorted EBV-exposed autologous B cells were cocultured with different numbers of flow-sorted NK cell subpopulations and IL-12 (10 ng/ml) for 10 d. EBV-transformed B cells were identified by the gating strategy shown in Fig. 5A. IR NK cells were 4.5-fold more efficient in preventing the outgrowth of EBV-infected B cells in vitro than were other tonsilar CD56^{bright} NK cells (Fig. 5B). These data provide direct evidence that the distinct IR NK cell subset has the ability to markedly restrict EBV-induced B cell transformation and is the optimum NK cell population in tonsils to perform this function.

Discussion

NK cells provide the first line of defense and play a crucial role against invading pathogens and emerging cancer cells (28–32). Our data demonstrate that these functions are mediated in part by these innate lymphocytes in SLOs and that the respective tonsilar IFN-γ^{high} NK cell subset is CD56^{bright}NKG2A^{+}CD94^{+}CD54^{+}CD62L^{-}, is present in tonsils ex vivo and is more mature than other CD56^{bright} NK cells in tonsils and less mature than other NK cells in peripheral blood, shows very low plasticity even after prolonged cytokine stimulation, accumulates in tonsils of EBV carriers, and is able to potently restrict EBV-induced transformation of B cells. Our findings suggest that a distinct NK cell subset is particularly operative against EBV at its portal of entry and could be exploited for the treatment of certain EBV-associated malignancies.

Our study indicates that EBV induces the accumulation of CD56^{bright}NKG2A^{+}CD94^{+}CD54^{+}CD62L^{-} NK cells with the capacity to produce high amounts of IFN-γ in human tonsils. These NK cells limit primary EBV infection of B cells more efficiently than do other tonsilar NK cell subsets and, therefore, could provide a crucial first innate barrier to infection in this portal organ of EBV entry. The reduced levels of IR cells in the blood of EBV^{+}

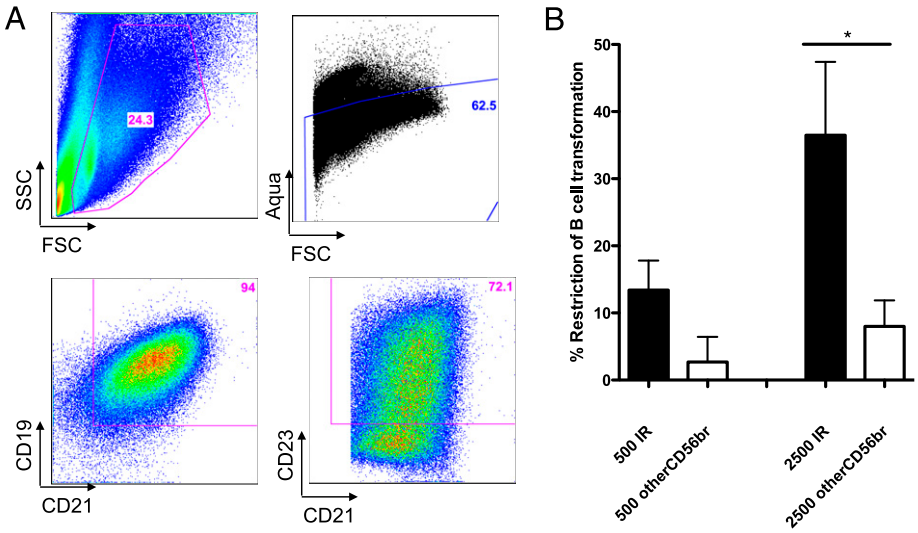

FIGURE 5. IR tonsilar NK cells potently restrict EBV-induced B cell transformation. Gating strategy for B cell analysis is shown in (**A**). (**B**) A total of 100,000 autologous B cells was infected with EBV and cocultured with the indicated numbers of sorted NK cells and IL-12 (10 ng/ml) for 10 d. The distinct IR subset has the ability to significantly restrict EBV-induced B cell transformation in physiological numbers (500 NK versus 100,000 B cells [i.e., 0.5% or a 5-fold increase]; 2,500 NK versus 100,000 B cells [i.e., 2.5%]). Gating strategy for sorted IR subset: lymphocyte population in FSC/SSC, live cells (Live/Dead cell stain Aqua$^-$), NK cells (CD3$^-$CD56$^+$); subset gating: CD56^{bright}NKG2A$^+$CD94$^+$CD54$^+$CD62L$^-$, as depicted in Supplemental Fig. 3A. Data from three independent experiments, with a total of four or five donors, are summarized. All error bars represent SEM. *$p \leq 0.05$ two-tailed nonparametric Mann–Whitney U test.

donors might be the result of recruiting or retaining this subset in tonsils, a further differentiation of this subset in the blood, or a combination of these factors. Enrichments of distinct NK cell populations were described for other viral infections in humans, but superior restriction of the respective pathogens by these subsets is rare. Moreover, a tissue-specific accumulation, as we describe for human tonsils, has not been reported previously.

Several studies showed persistent alterations in the NK cell repertoire after viral infections (13, 33, 34) and even protective effects of murine NK cell subsets (35, 36). Accumulation of terminally differentiated NK cell subpopulations were reported in hantavirus, chikungunya virus, human CMV, and HIV infections (13, 33, 37–39). These studies suggested that the respective viruses drive the accumulation of CD56^{dim}CD16$^+$KIR$^+$CD57$^+$ and often NKG2C$^+$ NK cells, which could mediate recognition of infected target cells or, alternatively, just differentiate in response to the inflammatory cytokines that are secreted during viral infections. Respectively, these expansions were proposed to reflect NK cells with memory-like features that prepare the NK cell compartment for more rapid secondary responses to recurrent or persistent viral infections (35, 39, 40) or accumulating effector cells that are activated by memory CD4$^+$ T cells via IL-2 secretion (41, 42). Very recently, cytokine-induced human memory-like NK cells were described (43). Both explanations for NK cell subset accumulation suggest that these innate lymphocyte populations should be explored for immunotherapy against the respective viral infections (e.g., by choosing adjuvants that would elicit cytokine production to promote in vivo differentiation of these NK cell populations or by adoptively transferring the respective NK cell subsets during symptomatic infection with the respective viruses and during dysregulation of persistent infections resulting in detrimental malignancies).

Such NK cell–based therapies have been explored successfully in transplantation settings against hematological malignancies. Several clinical centers have observed beneficial effects of haploidentical bone marrow transplantation for residual leukemia clearance after chemotherapy, harnessing NK cell–mediated graft-versus-leukemia effects (44–47). This therapy seems to be most effective when bone marrow donor and recipient are mismatched in HLA class I molecules, and the donor carries significant NK cell populations that can detect this mismatch via their KIR repertoire (46, 48) and then target leukemic cells by missing-self recognition (49). In these instances, transient reconstitution of NK cells with a donor phenotype before NK cell tolerance against the recipient is implemented mediates the graft-versus-leukemia effect. Alternatively, alloreactive NK cell populations that are expanded in vitro can also be infused (50–52). Thus, these studies provide good evidence that NK cell therapies that transiently generate an alloreactive compartment of these innate lymphocytes are well tolerated and could be readily expanded to harness specialized antiviral NK cell populations. The recent description of cytokine-induced human memory-like NK cells suggests that cytokine stimulation for expansion of NK cells before transfusion could be beneficial in these therapies (43). For the subset of potent IFN-γ–producing NK cells that can restrict EBV-induced B cell transformation, described in this article, we demonstrated an accumulation after IL-12 stimulation. Non-IR NK cells can change their phenotype accordingly after prolonged cytokine stimulation. This could be readily harnessed to enhance future NK cell–based therapeutic options by cytokine prestimulation.

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Disclosures

The authors have no financial conflicts of interest.

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